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13. ABSTRACT *(Maximum 200 Words)*

Steroid hormones, estrogen and progesterone, and their intracellular receptors play an important role in the development and progression of breast cancer. Coactivator proteins modulate the biological activity of these hormone receptors. We have cloned an E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP) as a coactivator of steroid hormone receptors. The purpose of this research is to explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer. We propose to explore this by developing animal models for overexpression and loss of function of E6-AP and then relate these observations to the clinical setting by studying the expression patterns of E6-AP in various human breast tumor biopsies. In this progress report, we report that we have successfully generated E6-AP overexpression models and an E6-AP null mouse line. Our data from these models suggest that overexpression of E6-AP in mammary gland results in impaired mammary gland development. Furthermore, loss of E6-AP expression results in an overly developed mammary gland compared to that of control mammary gland. These mice exhibit increased ductal branching and alveolar buds. However, the overexpression of transgene E6-AP and loss of function of E6-AP have no significant effects on the pregnant mammary glands. E6-AP possesses two independent and separable functions: coactivation and ubiquitin-protein ligase activity. Our data suggest that increased ductal branching and alveolar branching in E6-AP null mice are results of loss of ligase activity of E6-AP. In order to study the expression profile of E6-AP in human breast tumors, we examined 56 advanced stage human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of estrogen receptor-alpha in these tumors. Furthermore, our data also demonstrate that 83% human tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

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Introduction

Breast cancer is the leading cause of death in American women. It is anticipated that one woman out of ten will develop breast cancer at some point during her life (*1-6*). Although in recent years significant progress has been made in detection and treatment of the disease, much of the molecular basis of the disease remains unknown. This fact highlights the need to identify and understand the molecular basis associated with breast cancer development and progression.

Steroid hormones, estrogen and progesterone, play important role in the development and progression of breast cancer (*7-10*). Estrogens and progesterones exert their biological effects on target tissues through intracellular receptor proteins, estrogen (ER) and progesterone (PR) receptors (*11-13*). These receptors contain common structural motifs which include a less well conserved amino-terminal activation function (AF-1) that effects transcription efficiency, which has the hormone-independent activation function; a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determine target gene specificity; and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2); the region mediates the hormone-dependent activation function of the receptors (*11-13*).

In order to activate gene transcription, ER and PR undergo a series of well-defined steps. When bound to hormone, these receptors undergo a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with coactivators and recruitment of chromatin modifying enzyme activities such as histone acetyl transferase activity (HAT) and ATPase activity, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex (PIC) (*14-19*). These events are followed by up- or down-regulation of target gene expression.

Coactivators represent a growing class of proteins, which interact with receptors in a ligand-specific manner and serve to enhance their transcriptional activity. Prior to their identification, coactivators were predicted to exist based upon experiments, which showed that different receptors compete for a limiting pool of accessory factors required for optimal transcription. Stimulation of one receptor resulted in trans-repression of another receptor, indicating the depletion of a common coactivator pool (*20-22*). A number of coactivators have been cloned to date, including SRC-1 (*23*), TIF2 (GRIP1) (*24-27*), p/CIP (ACTR/RAC3/AIB1/TRAM-1) (*28-32*), PGCs (*33*), SRA (*34*), CBP (*35-37*) and **E6-associated protein (E6-AP)** (*38*) etc. and this list is growing rapidly day by day.

Coactivators were originally envisioned to serve a bridging role, linking the receptor to the basal transcription machinery (*39, 40*). Recently, the functional role of coactivators has expanded by the observation that they have been shown to possess enzymatic activities that may contribute to their ability to enhance receptor mediated transcription; SRC-1, p300/CBP, and ACTR (RAC3/AIB1) possess a histone acetyl transferase, HAT, activity (*14, 16, 28, 30, 41-43*) and members of SWI/SNF complex contain an ATPase activity (*44-47*). Ligand-activated receptors are thought to bring HAT and ATPase activities containing coactivators to the chromatin surrounding the receptor, disrupting the local repressive chromatin structure by acetylating histones and possibly other chromatin associated factors and catalyzing the uncoupling of ionic

interactions between histones and their substrate DNA (*42-48*). Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological responses to hormones (*17, 49-51*). The level of coactivator expression is critical in determining the activity of the receptor in target tissues and variations in hormone responsiveness seen in the population may be due to differences in coactivator levels.

It is accepted that coactivators either possess or bring HAT and ATPase activities to the promoter region of the target genes and presumably manifest part of their in vivo coactivation functions through these enzymatic activities (*42-47*). Recent identification of the enzymes of the ubiquitin-proteasome and ubiquitin-like pathways as coactivators by my own laboratory and others added a new twist to the coactivator field. These studies suggest that the ubiquitin-conjugating enzymes (UBCs) and the E3 ubiquitin-protein ligases, E6-AP and RPF1/RSP5, interact with members of the steroid hormone receptor superfamily including ER and PR and modulate their transactivation functions (*16, 38, 52-54*). Similarly, another coactivator protein, yeast SUG1, an ATPase subunit of the 26S-proteasome complex also interacts with and modulates steroid hormone receptor function (*55-57*). Instead of HAT activity, this group of coactivators possesses other enzymatic activities such as ubiquitin conjugation, ubiquitin ligation and protease activities. However, a common theme between the two groups of coactivators is that both possess some sort of enzymatic activity.

As mentioned above, my laboratory has identified ubiquitin pathway enzymes as coactivators of the nuclear hormone receptor superfamily. We have cloned an E3 ubiquitin-protein ligase, E6-AP as steroid hormone receptor interacting protein using a yeast two-hybrid screening assay. E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, PR, ER, androgen (AR) and glucocorticoid receptors (GR) (*38*). E6-AP was previously identified as a protein of 100 kDa, present both in the cytoplasm and the nucleus. E6-AP mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53, a growth-suppressive and tumor-suppressive protein. The E6/E6-AP complex specifically interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome protein degradation pathway (*58, 59*). As mentioned above, E6-AP is a member of the E3 class of functionally related ubiquitin-protein ligases. E3 enzymes have been proposed to play a major role in defining substrate specificity of the ubiquitin system (*60-62*). Protein ubiquitination also involves two other classes of enzymes, namely the E1 ubiquitin activating enzyme (UBA) and E2 ubiquitin conjugating enzymes, UBCs. The UBA first activates ubiquitin in an ATP-dependent manner. The activated ubiquitin then forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of the several E2s (UBCs), preserving the high-energy thioester bond. In some cases, ubiquitin is transferred directly from the E2 to the target protein through an isopeptide bond between the ε-amino group of lysine residues of the target protein and the carboxyl-terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin-protein ligase intermediate such as E6-AP (*63, 64*). The carboxyl-terminal 350 amino acids (aa) of E6-AP contains a "*hect*" (homologous to the E6-AP carboxy terminus) domain, which is conserved among all E3 ubiquitin protein-ligases and E6-AP related proteins characterized to date. The extreme carboxyl-terminal 100 aa contains the catalytic region of E6-AP, which transfers ubiquitin to the protein targeted for degradation (*60,*

61). We have shown that the ubiquitin-ligase activity of E6-AP is not required for the coactivation function of E6-AP. It has been shown that the conserved cysteine (C) 833 residue in E6-AP forms a thioester bond with ubiquitin and is necessary for the transfer of ubiquitin to the proteins targeted for ubiquitination. The mutation of C833 to alanine (A) or serine (S) has been shown to eliminate the ubiquitin-protein ligase activity of E6-AP (*60, 61*). In cotransfection studies, we showed that an E6-AP bearing a C-to-S mutation at the critical site was still able to coactivate steroid hormone receptors. Furthermore, our data also indicate that the catalytic function located within the *hect* domain of E6-AP is not necessary for the ability of E6-AP to interact with and coactivate steroid hormone receptor function, further confirming that the ubiquitin-ligase activity of E6-AP is not necessary for E6-AP to function as a coactivator (*38*). These findings indicate that E6-AP possesses two independent, separable functions, coactivation and ubiquitin-protein ligase activity (*38*).

It has been shown that altered expression of one nuclear receptor coactivator; AIB1 contributes to the development of hormone-dependent breast and ovarian cancer. Interaction of AIB1, SRC-1, TIF2, and p/CIP with CBP/ p300 is important for the coactivation function. Thus, overexpression or loss of expression of any of these coactivators could potentially perturb signal integration by CBP/ p300 and affect multiple transduction pathways (*28*). Recenly, it has also been shown that another steroid receptor coactivator, SRA is also elevated in breast tumors (*65*). Furthermore, recently, we have also shown that E6-AP is overexpressed 2.5-4.5 fold in 90-95% of tumors using a mouse mammary model of multistage tumorigenesis. E6-AP is overexpressed only in tumors but not in the intermediate steps of tumorigenesis (*66*).

The purpose of this research is to explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer. In the original proposal, we proposed to explore this by developing animal models for overexpression and loss of function of E6-AP. To relate the observations obtained from these animal models to the clinical setting, we also proposed to study the expression patterns of E6-AP in various human breast tumor biopsy samples. In this progress report, we report that we have successfully generated two E6-AP overexpression models. In these models, we have overexpressed wild-type E6-AP and ubiquitin-protein ligase defective mutant E6-AP (C833S) in the mammary epithelium of mouse by using the mouse mammary tumor virus (MMTV) promoter. In order to study the effect of loss of function of E6-AP on the normal mammary gland development and mammary gland tumor development, we have acquired an E6-AP null mouse line. These models will be helpful in understanding the role of E6-AP in the development and progression of breast tumors. Due to tropical storm Allison in June of 2001 we have lost 50% of our animals. This greatly hindered our progress. It took us six months to recover from this disaster. Presently, we are in the process of breeding and analyzing the mammary gland development of both the overexpression and the loss of E6-AP expression models. Our data from these models suggest that overexpression of E6-AP in mammary gland results in impaired mammary gland development. Furthermore, loss of E6-AP expression results in an overly developed mammary gland compared to that of the control mammary gland. These mutant mice exhibit increased ductal branching and alveolar buds. As mentioned above E6-AP contains both the coactivation activity and ubiquitin-protein ligase activity. In order to identify the role of E6-AP coactivation function versus its ubiquitin-protein ligase activity in the development of normal mammary gland and development of breast tumors, we also generated a mouse transgenic line, which overexpressed ubiquitin-protein ligase

defective and coactivation function intact E6-AP (C833S) in the mammary epithelium. Like E6-AP null mice, the overexpression of ubiquitin-protein ligase defective mutant E6-AP in mammary gland results in an overly developed mammary gland compared to that of the control mammary gland. Furthermore, these mice also exhibit increased ductal branching and alveolar buds. These data suggest that increased ductal branching and alveolar branching in E6-AP null mice are results of loss of ubiquitin-protein ligase activity of E6-AP. In order to study the expression profile of E6-AP in human breast tumors, we also examined 56 advanced stage human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of estrogen receptor-alpha in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. Furthermore, our data also demonstrate that 83% of human breast tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues and decrease in E6-AP expression is stage specific (stage IIB). These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

Body

In this original proposal, we hypothesized that E6-AP is an important modulator of the steroid hormone receptor mediated signal transduction pathway, and in cell growth and cycle control that are functionally significant in the development of breast cancer. In order to test this hypothesis we propose following objectives:

- **Development and analysis of animal models for the overexpression of wild-type E6-AP and ubiquitin-protein ligase defective mutant E6-AP in the mammary gland.**
- **Analysis of an animal model for loss of E6-AP expression in the mammary gland.**
- **Expression analysis of endogenous E6-AP, ER and p53 in human breast tumor biopsy samples.**

Development and analysis of an animal model for the overexpression of E6-AP in the mammary gland.

In order to test the effect of overexpression of E6-AP on the development of normal mammary gland and development of mammary tumors, we have successfully generated new transgenic mouse models. These models overexpress wild-type E6-AP and ubiquitin-protein ligase defective mutant E6-AP proteins in the mammary epithelium. In order to target the expression of E6-AP protein to the mammary epithelium we have utilized the MMTV promoter. Several other investigators have also successfully used this promoter to target expression of transgenes to the mammary gland (*67-70*).

Task 1. Design and generation of transgenic vectors

To overexpress wild-type E6-AP and ubiquitin-protein ligase defective mutant E6-AP in mouse mammary gland, transgenic expression vectors were generated (Fig. 1A). These vectors contain the MMTV promoter fused to either wild-type human E6-AP cDNA or to the ubiquitin-protein ligase defective (C833S) mutant E6-AP cDNA. To enhance the expression of the transgenes, the rabbit beta-globin gene fragment containing exon II, intron II, exon III and poly A signal

sequences were also incorporated into the transgene vectors. These vectors contain the requisite splice acceptor and donor sites for maximum transgene expression. In order to distinguish transgene expression from endogenous mouse E6-AP, we fused flag tag to the amino-terminus of the E6-AP. In the original proposal, we proposed to use the Anti-Express tag but it turns out that Anti-Express tag is not sensitive enough to detect the expression of E6-AP using Western blot analysis. Therefore, we were forced to incorporated flag tag in the E6-AP transgenic expression vector instead of Anti-Express tag. Using flag tag we have successfully detected the expression of wild-type E6-AP (Fig. 1B) and ubiquitin-protein ligase defective mutant E6-AP by Western blot analysis (data not shown).

The MMTV-flag-E6-AP transgenic expression vector was constructed as follows: initially a linker (5'-AATTCCCCGGG-3' and 5'-AATTCCCGGGG-3') containing the internal XmaI site was inserted into the E.CoRI site of the MMTVkBpA expression vector and resultant plasmid was named MMTVkBpAXmaI. To insert flag-tagged E6-AP into the MMTVkBpA expression plasmid, the full length E6-AP cDNA was amplified by polymerase chain reaction (PCR) with the primers containing flag tag sequences 5'-TCCCCCCGGGATGGACTACAAGGACGACGATGACAAGGAAGCCTGCACGAATGAG-3' (upper strand) and 5'-TCCCCCCGGGTTACAGCATGCCAAATCCTTTGGCATACGTGATGGCCTT-3' (lower strand). The PCR product was digested with XmaI and cloned into the corresponding site of the MMTVkBpAXmaI. After sub cloning the PCR amplified cDNA of E6-AP was sequenced and was found to be correct in sequence and reading frame. In order to make MMTV-mutant E6-AP (C833S), the full length cDNA of ubiquitin-protein ligase defective mutant E6-AP in which C833 was changed to S was amplified by PCR with the above mentioned primers and the PCR product was digested with XmaI and cloned into the corresponding site of the MMTVkBpAXmaI. After sub cloning the PCR amplified cDNA of E6-AP was sequenced and was found to be correct in sequence and reading frame.

In order to determine whether the MMTV-flag-E6-AP expression vectors was able to express full length E6-AP protein, this vector was transiently transfected into HeLa cells and the expression of E6-AP was detected by Western blot analysis using anti-flag tag specific antibodies. As shown in Fig. 1B, MMTV-flag-E6-AP expression vector was able to express full length E6-AP protein compared to that of control vector, which does not contain flag-E6-AP cDNA. Furthermore, Fig. 1B also suggest that anti-flag antibody is able to detect E6-AP expression by Western blot. Similarly, MMTV-mutant E6-AP expression vector was also able to express full length ubiquitin-protein ligase defective mutant E6-AP protein (data not shown).

Next we asked whether the wild-type flag-E6-AP protein and the ubiquitin-protein ligase defective mutant E6-AP protein functions as coactivator of nuclear hormone receptors. Previously, we have shown that E6-AP acts as a coactivator of PR and ER in cells. To test the coactivation function of wild-type flag-E6-AP and ubiquitin-protein ligase defective mutant E6-AP proteins, the E6-AP expression plasmids along with receptor expression and reporter plasmids were cotransfected into HeLa cells. Then cells were treated with appropriate hormones and the activity of the reporter gene was measured. Fig. 2 suggests that in the absence of ligand, PR has a minimal effect on reporter gene expression either in the absence or in the presence of E6-AP. Addition of hormone increases the reporter gene activity in the absence of E6-AP; when

flag-E6-AP was coexpressed with PR, the activity of PR was further stimulated by 4 to 5-fold. Similarly, the flag-E6-AP protein was also able to enhance the ER activity in HeLa cells (Fig. 3). Like wild-type E6-AP, the ubiquitin-protein ligase defective mutant E6-AP was also able to enhance both the PR and ER activities. These data suggest that wild-type flag-E6-AP and ubiquitin-protein ligase defective mutant E6-AP are functional and both act as coactivators of PR and ER.

Task 2. Generation of transgenic animals

After establishing that the wild-type flag-tagged E6-AP protein is intact and biologically functional, the transgene was released from the transgenic expression vector by digesting MMTV-flag-E6-AP vector with NotI and KpnI enzymes. After purification from the vector backbone, the transgene DNA was extracted with phenol-chloroform and ethanol pracipitated. After precipitation, the transgene DNA was suspended in injection buffer and microinjected into fertilized FVB one-cell embryos. The injected embryos were then implanted into the oviducts of pseudopregnant recipient mothers. Once, animals were born, the transgenic founders were identified by PCR and/or Southern blot analysis.

Task 3. Identification of transgenic founders

In order to identify the transgenic lines, we have developed a PCR screening method. PCR screening is faster and cheaper compared to Southern blot screening. To develop PCR screen we designed 2 pairs of primer sets. The locations of these primers in the transgene are shown in Fig. 4. The primers 1 and 2 will generate a 385bp fragment and while the primers 3 and 4 will generate 450bp fragment (Fig. 4). The transgene-negative animals will not generate these bands. The sequence of the primers are as follows: primer 1, 5'-TGCTAACCATGTTCATGCC-3'; primer 2, 5'-CTCAGAGCAGGAGTTGTTGGG-3'; primer 3, 5'-ATGGACTACAAGGACGACGATG-3' and primer 4 5'-CCGGAAGCTCTGTACC-3'. In order to confirm the PCR result we have also performed Southern blot analysis of transgenic lines (Fig. 5). The lines, which are positive for transgene by PCR method, are also positive by Southern blot analysis (Fig. 5). By using PCR and Southern blot screening methods, we identified two transgene positive founder lines. However, only one founder was able to transmit the transgene to the offsprings. After another round of injection, we have identified two more transgenic lines. In total we have three transgene lines which can transmit transgene to their offspring. Finally, founders were bred with wild-type FVB mice to generate female mice for further analyses.

Task 4 and 5. Breed founders and analysis of expression patterns of transgene

Next, we analyzed transgenic lines for the expression of transgene human E6-AP in the mouse mammary gland. In order to confirm whether the human E6-AP transgene is expressed in mouse mammary gland, we analyzed the mammary glands of 8 weeks old virgin female mice by immunohistochemistry using an anti-E6-AP specific antibody obtained from Dr. N. J. Maitland. As a control, we also analyzed the mammary glands of the age matched wild-type non-transgenic animals. In order to study the expression profile of transgene, the mammary glands from 8 weeks old wild-type non-transgenic and transgenic virgin female mice were microdissected,

fixed in 10% formalin and processed for immunohistochemistry studies using an anti-E6-AP specific polyclonal antibody produced in rabbit. This antibody recognized both human E6-AP and endogenous mouse E6-AP. As shown in Fig. 6, the human E6-AP transgene is highly expressed in the transgenic line E106 (Fig. 6A) and moderately expressed in transgenic lines E95 (Fig. 6B) and E37 (data not shown). Furthermore, transgene is specifically targeted to the mammary epithelium. Fig. 6 also suggests that the expression of endogenous mouse E6-AP is very low in the mammary gland. The control sections incubated with normal serum showed no signal (data not shown). We have also analyzed the expression of transgene by using anti-flag antibody. This analysis also demonstrates that human E6-AP transgene is highly expressed in the transgenic line E106 and moderately expressed in transgenic lines E95 and E37 (data not shown). These results were also further confirmed by Western blot analysis (data not shown).

To determine the tissue specificity of transgene expression, total cellular extracts were prepared from various transgenic tissues (brain, liver, mammary gland and heart) and the expression pattern of transgene was compared with that of non-transgenic tissues by Western blot analysis using anti-E6-AP antibody. As expected endogenous E6-AP is highly expressed in brain and liver (Fig. 7). The expression of E6-AP is moderate in heart. However, low expression of endogenous E6-AP was detected in mammary gland. Fig. 7 shows that transgene human E6-AP is selectively overexpressed in mammary gland. However, the transgenic line E37 also showed some expression of transgene in lung (data not shown). The expression profile of our transgene is in agreement with that of published expression profile of other MMTV-transgenes. Again, transgenic line E106 showed highest expression of transgene and transgenic line E95 showed moderate expression of transgene (Fig. 7).

Task 6. Morphological and histological analysis of transgenic mammary glands

In order to analyze for physiological perturbations that could be attributed to the overexpression of E6-AP, we performed whole-mount analysis of transgenic and age-matched wild-type non-transgenic mammary glands at different stages of development, (a) virgin (8 weeks old, 12 weeks old and 68 weeks old); (b) pregnant; (c) lactation; and (d) involution (15 days involuting and 8 weeks involuting) stages. In newborn mice, the mammary gland is comprises of a few ducts and it undergoes extensive growth post-natally. During puberty, the elongation and arborization of the ducts progress gradually into the surrounding mammary fatpad under the influence of gonadal hormones and terminate at the limits of the fatpad. With each subsequent estrous cycle, the lateral ductal branches subdivide progressively and give rise to small alveolar buds. During pregnancy additional ductal branching occurs and extensive lobular-alveolar proliferation gradually results in the complete filling of the fat pad at parturition (*71*). The whole mount analyses of 8 weeks old, 12 weeks old and 68 weeks old virgin mammary glands from human E6-AP transgenic lines (E95 and E106) and wild-type non-transgenic lines were performed. As shown in Fig. 8, 9 and 10, overexpression of transgene human E6-AP results in impaired mammary gland development compared to wild-type non-transgenic litttermates. The transgenic mammary glands failed to invade the entire fat pad. In contrast, the wild-type gland was able to invade the entire fat pad. In this study we used lymph node as a reference point to evaluate ductal outgrowth. In each group at least six animals were analyzed.

Next, we ask whether overexpression of transgene human E6-AP has any effect on the pregnant mammary gland. Again, we performed whole mount analysis on 15 days pregnant mammary gland from transgenic and non-transgenic mice. As shown in Fig. 11, the overexpression of human E6-AP has no significant effect on the pregnant mammary gland. The phenotype of the pregnant transgenic and non-transgenic mammary glands is identical. Both glands have identical lobular-alveolar proliferation.

In order to study the effects of overexpression of transgene human E6-AP on the involution process of the mammary gland, we analyzed 15 days involuting mammary glands from both transgenic and non-transgenic mice. Fig. 12 suggests that like pregnant glands, the transgene have no significant effect on the involution process during first 15 days. The involution is identical in transgenic and non-transgenic glands during first 15 days. In contrast, whole mount analysis of 8 weeks old involuting transgenic and non-transgenic mammary glands suggest that the transgenic gland involute faster than nontransgenic gland (Fig. 13). Like virgin transgenic mammary gland, the 8 weeks old involuting transgenic gland is smaller compared to that of non-transgenic gland (Fig. 13).

Task 7. Analysis of p53 in transgenic mammary glands

The expression analysis of p53 in transgenic mammary glands is in progress.

Analysis of an animal model for loss of E6-AP expression in the mammary gland.

The second aim of this proposal is to test the effects of loss of steroid hormone receptor coactivator, E6-AP, on the normal development of mammary gland and mammary gland tumors. In order to study the effect of loss of function of E6-AP on the normal breast development and breast tumor development, we have acquired an E6-AP null mouse line in our laboratory. Dr. Aurther Beaudet at the Baylor College of Medicine generated this line (*72*).

Task 8. Screening and breeding of E6-AP null mutant mice

To screen for the E6-AP null animals, we have also developed a PCR screening method. In order to develop the PCR screening method, we have designed 3 primers, which can differentiate between wild-type and null E6-AP locus. The sequence of the primers are: primer 1, 5'-ACTTCTCAAGGTAAGCTGAGCTTGC-3'; primer 2, 5'-GCTCAAGGTTGTATGCCTTGGTGCT-3' and primer 3, 5'-TGCATCGCATTGTCTGAGTAGGTGTC-3'. By using these 3 primers, we have successfully amplified the 750 bp fragment of wild-type E6-AP allele and 350 bp fragment of E6-AP null allele, respectively. The wild-type animals have only band of 750 bp, whereas homozygous E6-AP null animals have only 350 bp amplified fragment and heterozygous animals contain both bands (Fig. 14).

Task 9. Morphological and histological analysis of E6-AP null mutant mammary glands

In order to study the consequences of the loss of E6-AP expression on mammary gland development, the whole mount analyses of 8 weeks old and 12 weeks old virgin mammary glands from E6-AP null and wild-type mice were performed. As shown in Fig. 15 and 16A and B, loss of E6-AP results in increased lobular-alveolar buds compare to wild-type normal mammary glands. The E6-AP null mammary glands are more developed and the degree of mammary gland development is similar to that of a 5-10 days pregnant wild-type mammary gland. Increased lobular-alveolar buds in E6-AP null mammary glands compare to that of wild-type mammary glands are clearly visible at higher magnification in Fig. 16B.

Next, we ask whether loss of E6-AP expression has any effect on pregnant mammary gland. Again, we performed whole mount analysis on 15 days pregnant mammary gland from E6-AP null and normal wild-type mice. As shown in Fig. 17, the loss of E6-AP expression has no significant effect on the pregnant mammary glands. The phenotype of the pregnant E6-AP null and wild-type mammary glands is identical. Both glands have identical lobular-alveolar proliferation.

In order to study the effects of loss of E6-AP expression on the involution process of the mammary gland, we analyzed 15 days involuting mammary glands from both E6-AP null and wild-type mice. Fig. 18A and B suggests that like pregnant glands, the loss of E6-AP expression have no significant effect on the involution process. The involution is identical in transgenic and non-transgenic glands.

Task 10. Analysis of p53 expression in E6-AP null mutant mammary glands

The expression analysis of p53 in E6-AP null mutant mammary glands is in progress.

Development and analysis of an animal model for the overexpression of ubiquitin-protein ligase defective mutant E6-AP (C833S) in the mammary gland.

E6-AP possesses two independent and separable functions: coactivation and ubiquitin-protein ligase activity. Identification of the role of E6-AP coactivation function versus its ubiquitin-protein ligase activity in the development of normal mammary gland and development of mammary tumors would be critical to understand the molecular pathway by which E6-AP exert its effects. In order to identify the role of E6-AP coactivation function versus its ubiquitin-protein ligase activity in the development of normal mammary gland and development of breast tumors, we also generated a mouse transgenic line, which overexpress ubiquitin-protein ligase defective and coactivation function intact E6-AP (C833S) in the mammary epithelium.

Task 11. Generation of transgenic animals

In order to generate mouse transgenic lines that overexpress the ubiquitin-protein ligase defective mutant E6-AP, the transgene was released from the transgenic expression vector by digesting MMTV-mutant-E6-AP vector with NotI and KpnI enzymes. After purification from the vector backbone, the transgene DNA was extracted with phenol-chloroform and ethanol pracipitated. After precipitation, the transgene DNA was suspended in injection buffer and microinjected into fertilized FVB one-cell embryos. The injected embryos were then implanted into the oviducts of

pseudopregnant recipient mothers. Once, animals were born, the transgenic founders were identified by PCR and/or Southern blot analysis.

Task 12. Breed founders and analysis of expression patterns of ubiquitin-protein ligase defective mutant E6-AP transgene

In order to confirm whether the ubiquitin-protein ligase defective mutant E6-AP transgene is expressed in mouse mammary gland, we analyzed the mammary glands of 8 weeks old virgin female mice by immunohistochemistry using an anti-E6-AP specific antibody. As a control, we also analyzed the mammary glands of the age matched wild-type non-transgenic animals. In order to study the expression profile of transgene, the mammary glands from 8 weeks old wild-type non-transgenic and transgenic virgin female mice were microdissected, fixed in 10% formalin and processed for immunohistochemistry studies using an anti-E6-AP specific polyclonal antibody. As shown in Fig. 19, the ubiquitin-protein ligase defective mutant E6-AP transgene is highly expressed in the transgenic line (Fig. 19). Furthermore, our data also demonsttrate that the ubiquitin-protein ligase defective mutant E6-AP transgene is specifically targeted to the mammary epithelium (data not shown). Furthermore, transgene is selectively overexpressed in mammary gland.

Task 13. Morphological and histological analysis of transgenic mammary glands

In order to analyze for physiological perturbations that could be attributed to the overexpression of ubiquitin-protein ligase defective mutant E6-AP, we performed whole-mount analysis of transgenic and age-matched wild-type non-transgenic mammary glands at different stages of development, (a) virgin (12 weeks old); (b) pregnant; (c) lactation; and (d) involution (15 days involuting and 8 weeks involuting) stages. As shown in Fig. 20A and B, like E6-AP null mice, the overexpression of ubiquitin-protein ligase defective mutant E6-AP in mammary gland results in an overly developed mammary gland compared to that of the control mammary gland. Furthermore, these mice also exhibit increased ductal branching and alveolar buds. Increased lobular-alveolar buds in mutant E6-AP transgenic mammary glands compare to that of wild-type mammary glands are clearly visible at higher magnification in Fig. 20B. These data suggest that increased ductal branching and alveolar branching in E6-AP null mice are results of loss of ubiquitin-protein ligase activity of E6-AP.

Next, we ask whether overexpression of ubiquitin-protein ligase defective mutant E6-AP transgene has any effect on the pregnant mammary gland. Again, we performed whole mount analysis on 15 days pregnant mammary gland from transgenic and non-transgenic mice. As shown in Fig. 21, the overexpression of ubiquitin-protein ligase defective mutant E6-AP has no significant effect on the pregnant mammary gland. The phenotype of the pregnant transgenic and non-transgenic mammary glands is identical. Both glands have identical lobular-alveolar proliferation.

In order to study the effects of overexpression of ubiquitin-protein ligase defective mutant E6-AP transgene on the involution process of the mammary gland, we analyzed 15 days involuting mammary glands from both transgenic and non-transgenic mice. Fig. 22 suggests that like

pregnant glands, the transgene have no significant effect on the involution process during first 15 days. The involution is identical in transgenic and non-transgenic glands during first 15 days. In contrast, whole mount analysis of 8 weeks old involuting transgenic and non-transgenic mammary glands suggest that the transgenic gland has increased alveolar buds compared to that of nontransgenic gland (Fig. 23). Like virgin E6-AP null mammary gland, the 8 weeks old involuting transgenic gland has more lobular-alveolar buds compared to that of non-transgenic gland (Fig. 23).

Expression analysis of endogenous E6-AP, ER and p53 in human breast tumor biopsy samples.

The third aim of the proposal is to test the expression of endogenous E6-AP, ER and p53 in human breast tumor biopsies. To date we have examined expression levels of E6-AP and ER in 56 different breast tumors and expression of p53 in 20 different tumors.

Task 14. Expression analysis of endogenous E6-AP and p53

To study the expression profile of E6-AP in human breast tumors, to date we have examined 56 advanced stage human breast cancer biopsy samples by Western blot using E6-AP specific antibody. Fig. 24 shows the expression of E6-AP in 20 different tumor samples. Majority of the tumors expresses E6-AP. We have also confirmed these results by immunofluorescent method using an anti-E6-AP antibody. Since, E6-AP is an E3 ubiquitin-protein ligase enzyme and recently, we have shown that ER is degraded through the ubiquitin proteasome pathway. Therefore, we also analyzed the expression profile of ER in breast tumors (Fig. 24) and then compare it with that of E6-AP expression (Fig. 25). We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant (Fig. 25).

It has been demonstrated that E6-AP promotes the degradation of p53 via the ubiquitin degradation pathway. Furthermore, in the brain of E6-AP knockout animals, the protein levels of p53 accumulate compared to those of normal littermates. Therefore, we also analyzed the endogenous expression of p53 protein from breast tumor biopsies. As shown in Fig. 24, p53 expression was not detectable in most tumors except tumor number 7, 10, 13 and 15. Furthermore, there was no statistical correlation between the expression profile of E6-AP and p53.

We have also examined the expression profile of E6-AP in normal human mammary tissues by immunohistochemistry using anti-E6-AP antibody. Fig. 26 suggests that E6-AP is highly expressed in normal human mammary ducts and almost every epithelial cell express E6-AP protein (Fig. 26).

In order to study the expression profile of E6-AP in human breast tumor samples, we examined the expression profile of E6-AP in 36 different human breast tumor samples and adjacent normal tissues by immunohistochemistry. As mentioned above, E6-AP expression is very high in

normal mammary ducts (Fig. 27). However, 83% of human tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues (Fig. 27).

Next, we analyze the expression level of E6-AP between different stages of breast cancer by immunohistochemistry and then compare the expression profile of E6-AP within different stages by Wicoxonrank-sum test. This analysis suggest that E6-AP expression is decreased in stage IIB breast tumors (Fig. 28).

Statement of work accomplished/in progress

Task 1. Design and generation of transgenic vectors. **Accomplished.**
Task 2. Generation of transgenic animals that overexpress wild-type E6-AP. **Accomplished.**
Task 3. Identification of transgenic founders. **Accomplished.**
Task 4. Breed founders to assess expression and expand positive lines. **Accomplished.**
Task 5. Analysis of expression patterns of transgene in different development stages of mammary glands. **In Progress.**
Task 6. Morphological and histological analysis of transgenic mammary glands. **In Progress.**
Task 7. Analysis of p53 expression in transgenic mammary glands. **In Progress.**
Task 8. Breeding of E6-AP null mutant mice. **Accomplished**
Task 9. Morphological and histological analysis of E6-AP null mutant mammary glands. **In Progress.**
Task 10. Analysis of p53 expression in E6-AP null mutant mammary glands. **In Progress.**
Task 11. Generation of transgenic animals that overexpress ubiquitin-protein ligase defective mutant E6-AP. **Accomplished.**
Task 12. Breed founders and analysis of expression patterns of ubiquitin-protein ligase defective mutant E6-AP transgene. **Accomplished.**
Task 13. Morphological and histological analysis of transgenic mammary glands. **In Progress.**
Task 14. Expression analysis of endogenous E6-AP, ER and p53. **Accomplished.**

Key Research Accomplishments

- E6-AP transgenic expression vectors have been generated
- Expression analysis of E6-AP from the transgenic vector has been completed
- The biological activity of the wild-type flag-E6-AP and ubiquitin-protein ligase defective mutant E6-AP have been analyzed
- Wild-type E6-AP transgenic mouse lines have been generated
- PCR based screening method for identification of transgenic animals has been developed
- E6-AP transgenic founders have been identified by both PCR and Southern blot
- Expression analysis of transgenes have been analyzed in mammary glands
- The tissue specificity of transgenes have been analyzed
- Analysis of E6-AP transgenic mammary glands has been done in virgin, pregnant and involution stages
- E6-AP knockout animals have been acquired
- PCR based screening method for identification of E6-AP knockout animals has been developed

- Analysis of E6-AP null mammary glands has been done in virgin, pregnant and involution stages
- Ubiquitin-protein ligase defective mutant E6-AP transgenic lines have been generated
- Expression analysis of the ubiquitin-protein ligase defective mutant E6-AP transgenes have been analyzed in mammary glands
- The tissue specificity of the ubiquitin-protein ligase defective mutant E6-AP transgenes have been analyzed
- Analysis of the ubiquitin-protein ligase defective mutant E6-AP transgenic mammary glands has been done in virgin, pregnant and involution stages
- Expression analysis of E6-AP in 56 different tumors has been done
- Expression analysis of ER in 56 different tumors has been done
- Expression profile of E6-AP has been compared with that of ER expression
- Expression analysis of p53 in 20 different tumors has been done
- Expression analysis of E6-AP by immunohistochemistry has been done in 36 different breast tumor samples
- Expression analysis of E6-AP in different tumor stages has been done
- Expression profile of E6-AP has been compared within different tumor stages

Reportable Outcomes

The ongoing work described here is going to be presented as a poster and an abstract at the Annual Endocrine Society Meeting (June 2002), in San Francisco, California (see appendix 2). Furthermore, this work has also been accepted for publication in Breast Cancer Research (see appendix 2).

Conclusions

We have successfully generated an E6-AP overexpression model. In order to study the effect of loss of function of E6-AP on the normal breast development and breast tumor development, we have acquired an E6-AP null mouse line. Presently, we are in the process of breeding and analyzing the mammary gland development of both the overexpression and the loss of E6-AP expression models. Our data from these models suggest that overexpression of E6-AP in mammary gland results in impaired mammary gland development. Furthermore, loss of E6-AP expression results in an overly developed mammary gland compare to that of controls mammary gland. These mice exhibit increased ductal branching and alveolar buds. E6-AP possesses two independent and separable functions: coactivation and ubiquitin-protein ligase activity. Our data suggest that increased ductal branching and alveolar branching in E6-AP null mice are results of loss of ligase activity of E6-AP. In order to study the expression profile of E6-AP in human breast tumors, we examined 56 advanced stage human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of estrogen receptor-alpha in these tumors. Furthermore, our data also demonstrate that 83% human tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

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Appendices

1. Figures 1-28

2. 1 Abstract and 1 Manuscript

Appendix 1

Figures 1-28

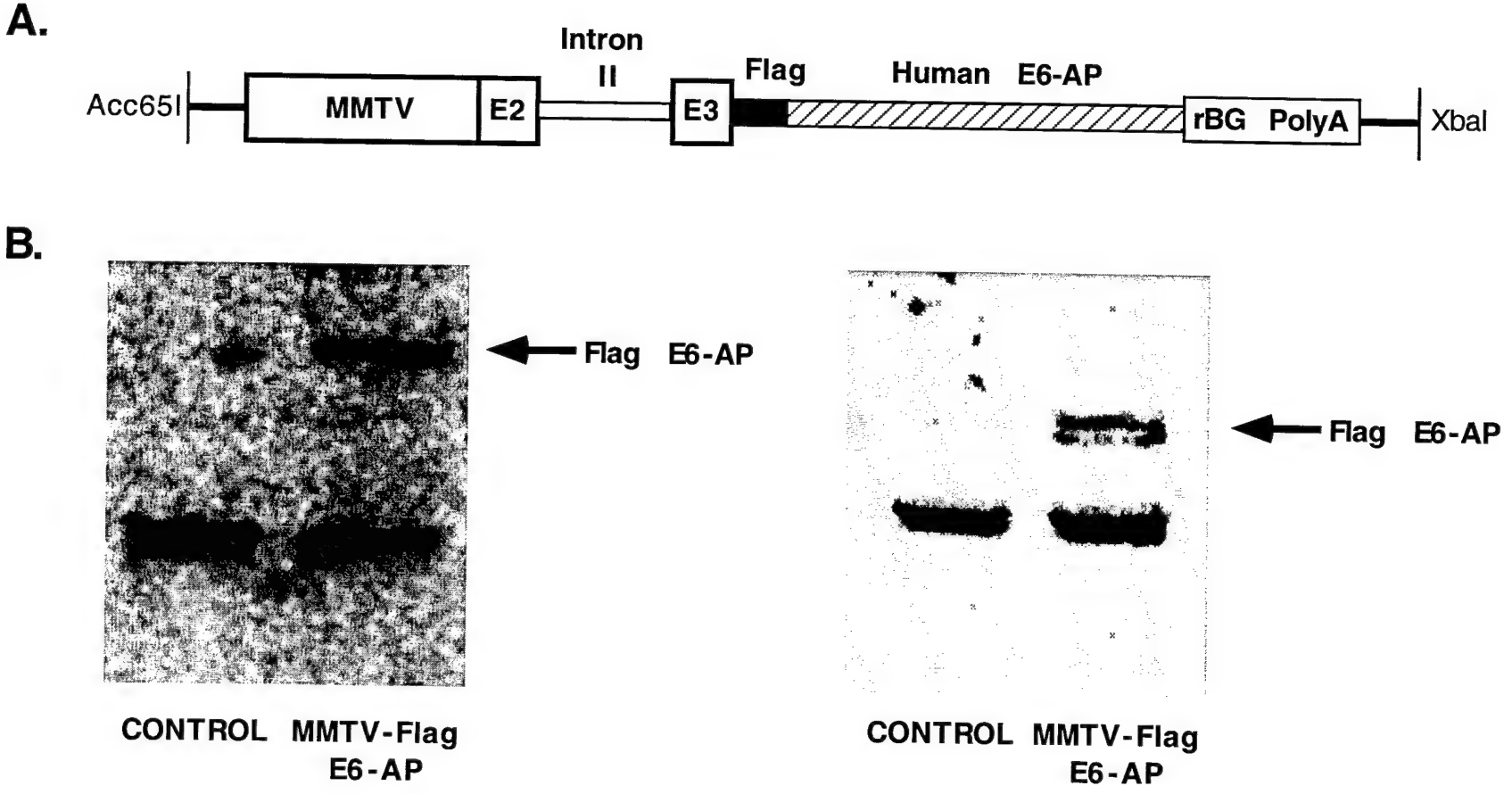


Figure 1: Generation and characterization of MMTV-E6-AP transgene. **A.** The MMTV-E6-AP construct contains the MMTV promoter and the full-length human E6-AP cDNA, fused to the exon II (E2), intron II, exon III (E3) and the rat beta-globin gene polyadenylation signal (rBG PolyA). **B.** HeLa cells were transiently transfected with either control plasmid or MMTV-Flag-E6-AP expression plasmid and the expression of E6-AP was detected by Western blot analysis using anti-flag tag specific antibody.

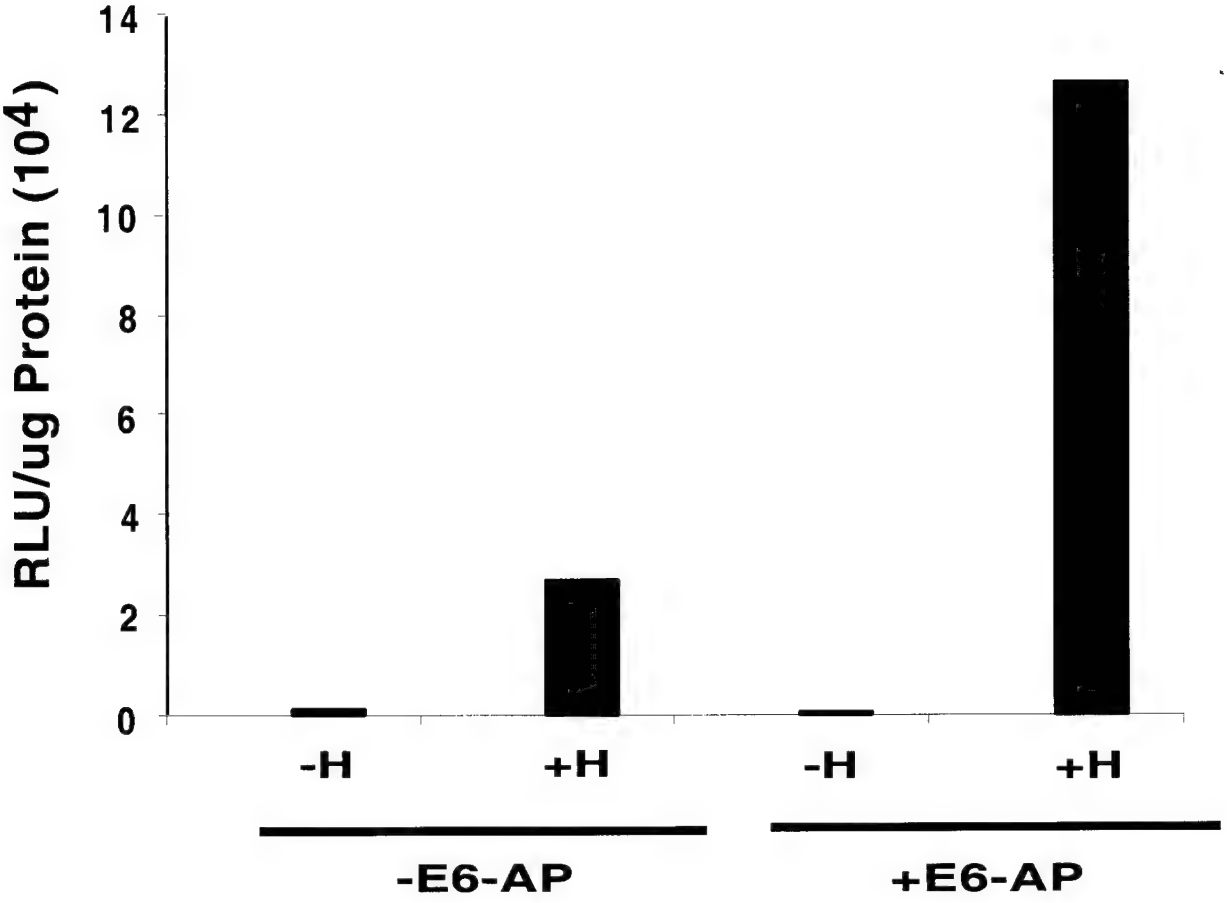


Figure 2: MMTV-Flag-E6-AP Coactivates PR Activity. Hela cells were transiently transfected with progesterone receptor expression plasmid and progesterone-responsive reporter plasmid in the absence or presence of E6-AP expression plasmid. The cells were treated with either vehicle (-H) or 10^{-7}M progesterone (+H). The data is presented as relative light units/ug protein (RLU/ug Protein).

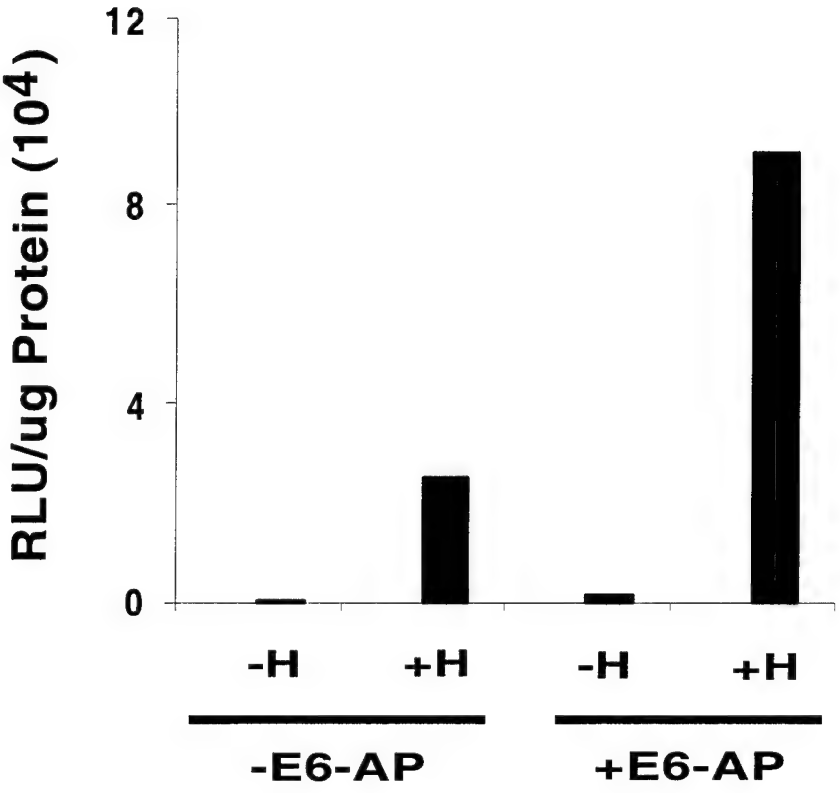


Figure 3: MMTV-Flag-E6-AP Coactivates ER Activity. Hela cells were transiently transfected with estrogen receptor expression plasmid and estrogen-responsive reporter plasmid in the absence or presence of E6-AP expression plasmid. The cells were treated with either vehicle (-H) or 10^{-7}M estradiol (+H). The data is presented as relative light units/ug protein (RLU/ug Protein).

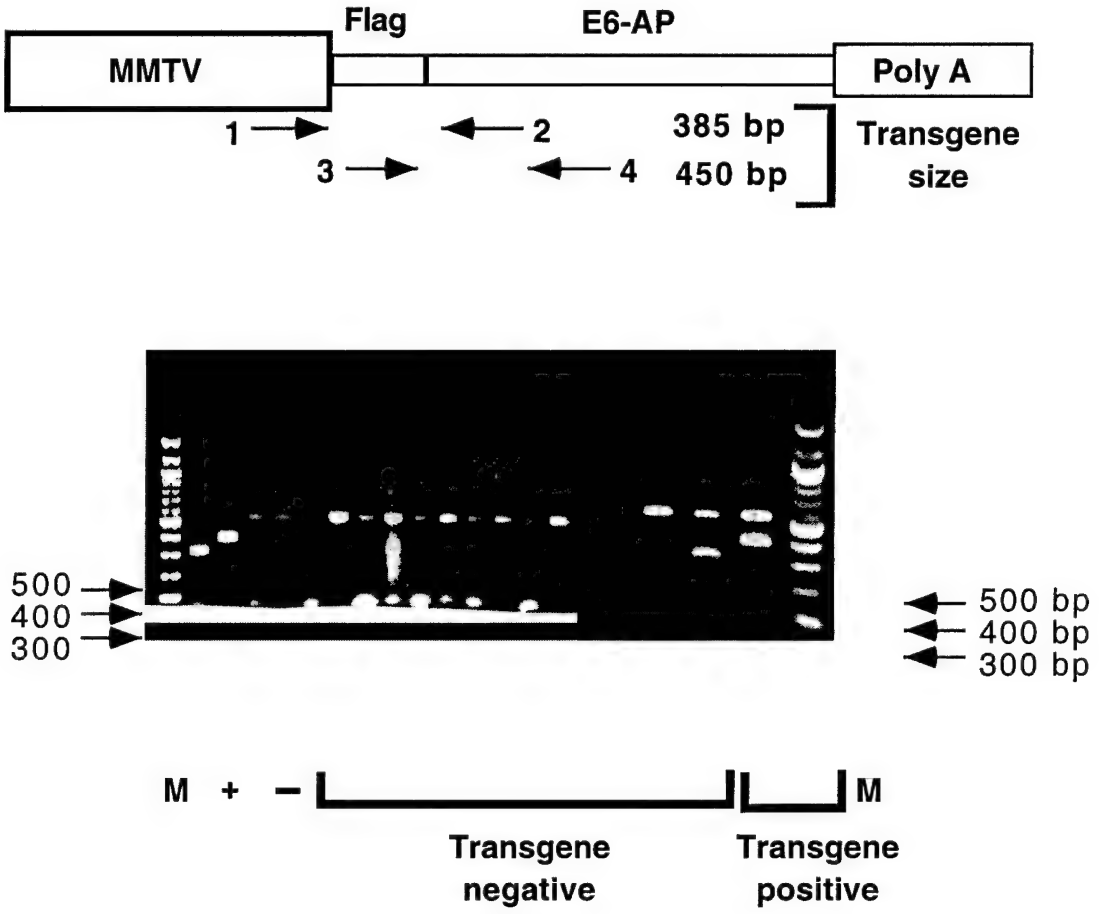


Figure 4: Screening of E6-AP transgenic lines by PCR method.
In order to identify the transgenic lines, a PCR screening method was developed . To develop PCR screen 2 pairs of primer sets were designed. The locations of these primers in transgene are shown in by arrows. The primers 1and 2 amplify a 385bp fragment and the primers 3 and 4 generate 450bp fragment. The transgene negative animals did not generate these bands.

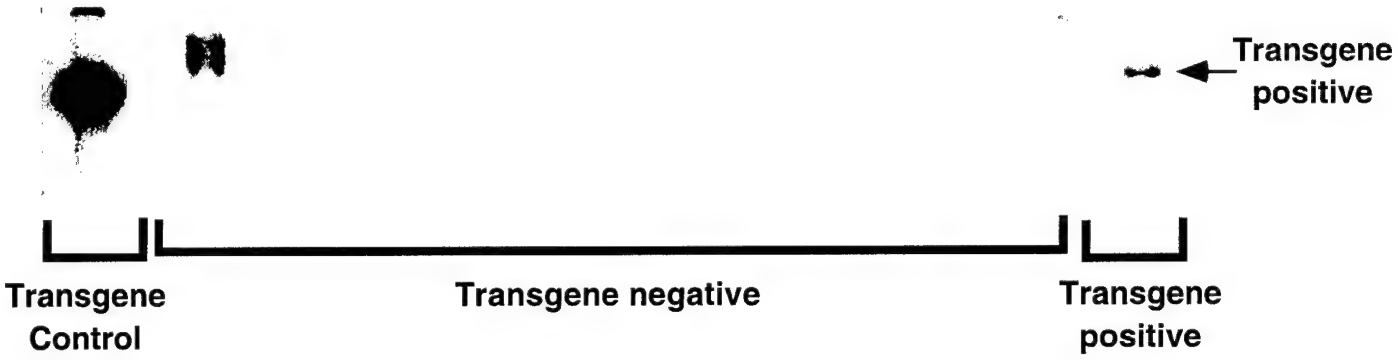
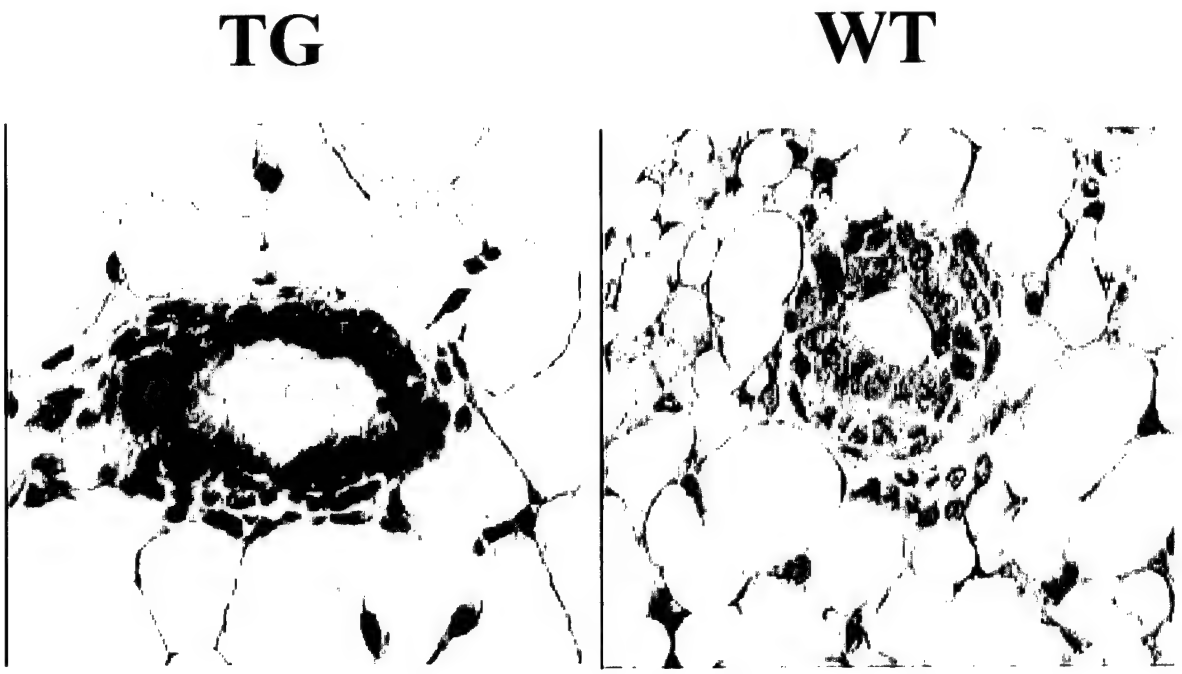


Figure 5: Screening of E6-AP transgenic lines by Southern blot.
Total genomic DNA was isolated from mice tails. The Southern blot was performed by using the 700 bp (BamHI-E.CoRI) long fragment of E6-AP as probe. The genomic DNA was digested with BamHI and BglII.

A.



B.

Figure 6: Expression analysis of transgene (MMTV-E6-AP) in the mouse mammary glands. E6-AP expression in the mouse mammary glands was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal is seen as dark (brown) spots. WT, Wild-type mammary gland; TG, E6-AP transgenic mammarygland. A. Transgenic line E106. B. Transgenic line E95.

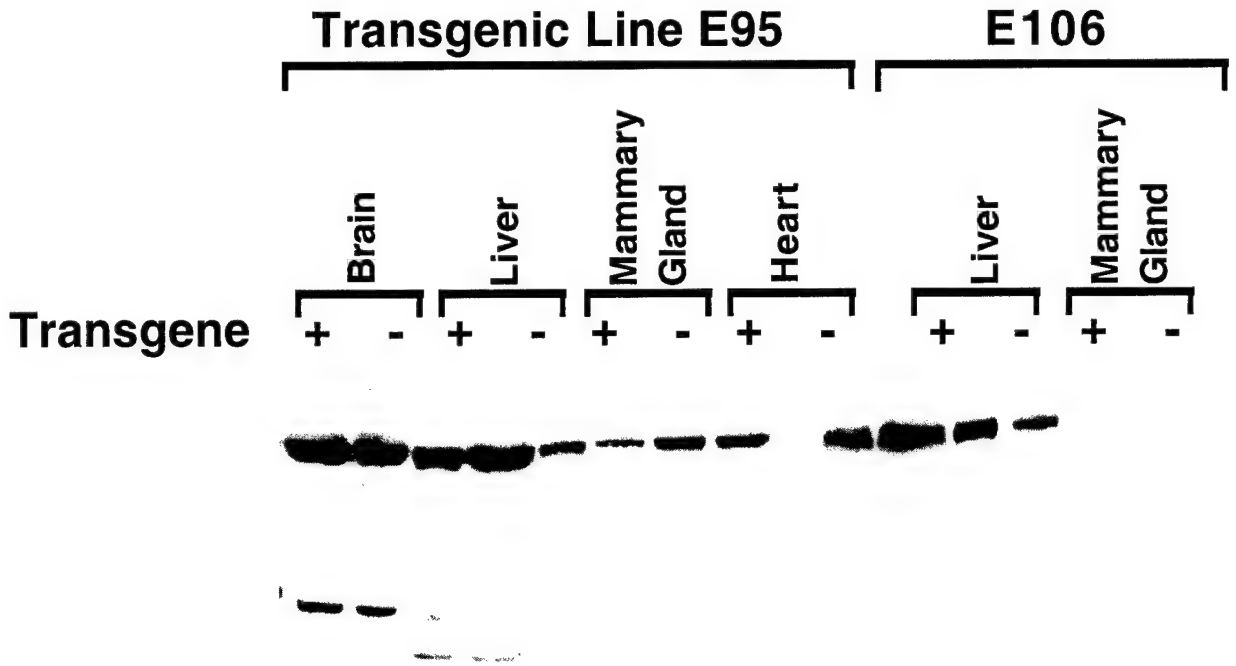


Figure 7: MMTV-driven E6-AP transgene preferentially overexpressed in mammary gland. Expression analysis of endogenous E6-AP and MMTV-driven human E6-AP transgene was performed in various mouse tissues such as brain, liver, mammary gland, heart etc. by Western blot using E6-AP specific antibody. MMTV-driven E6-AP transgene is specifically overexpressed in mammary gland.

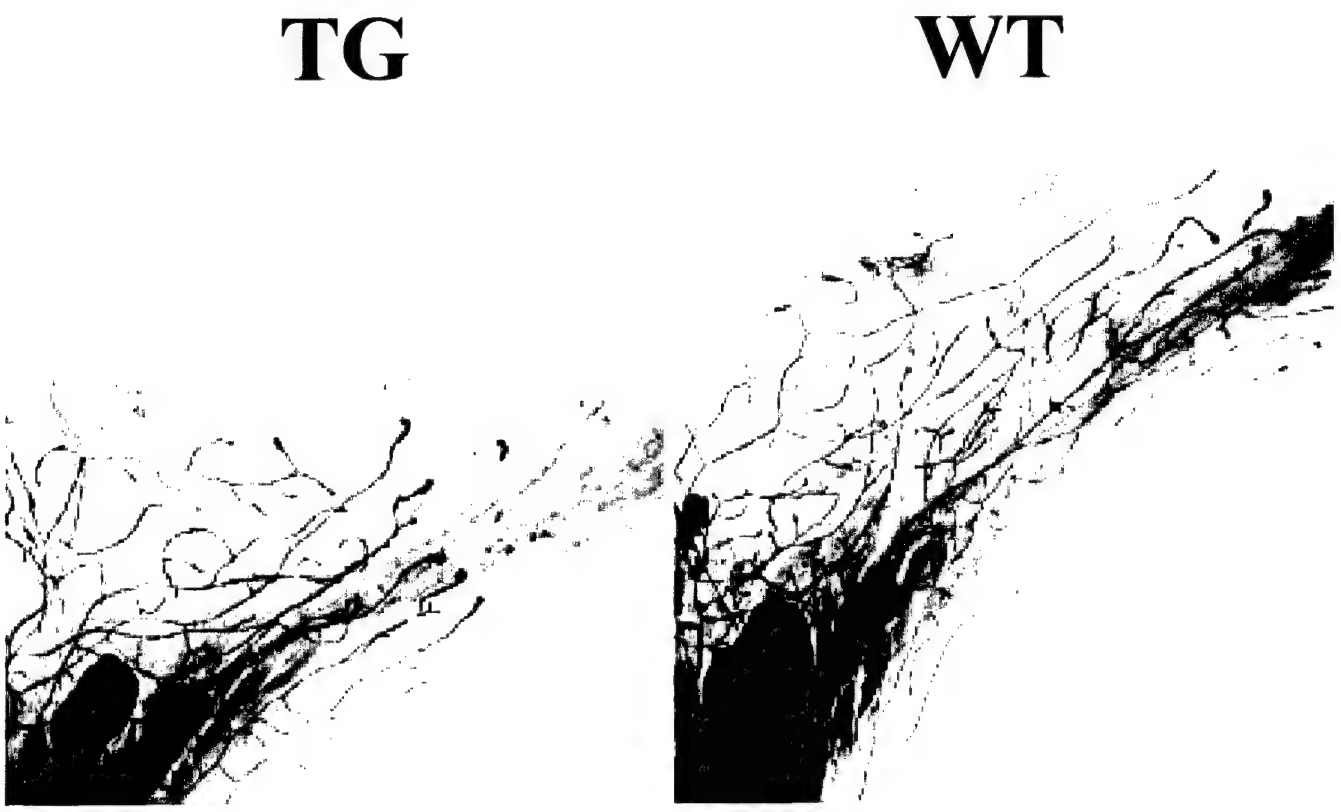


Figure 8: Overexpression of human E6-AP in mouse mammary gland results in impaired mammary gland development. Whole mount analyses of mammary glands from 8 weeks old virgin mice were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG).

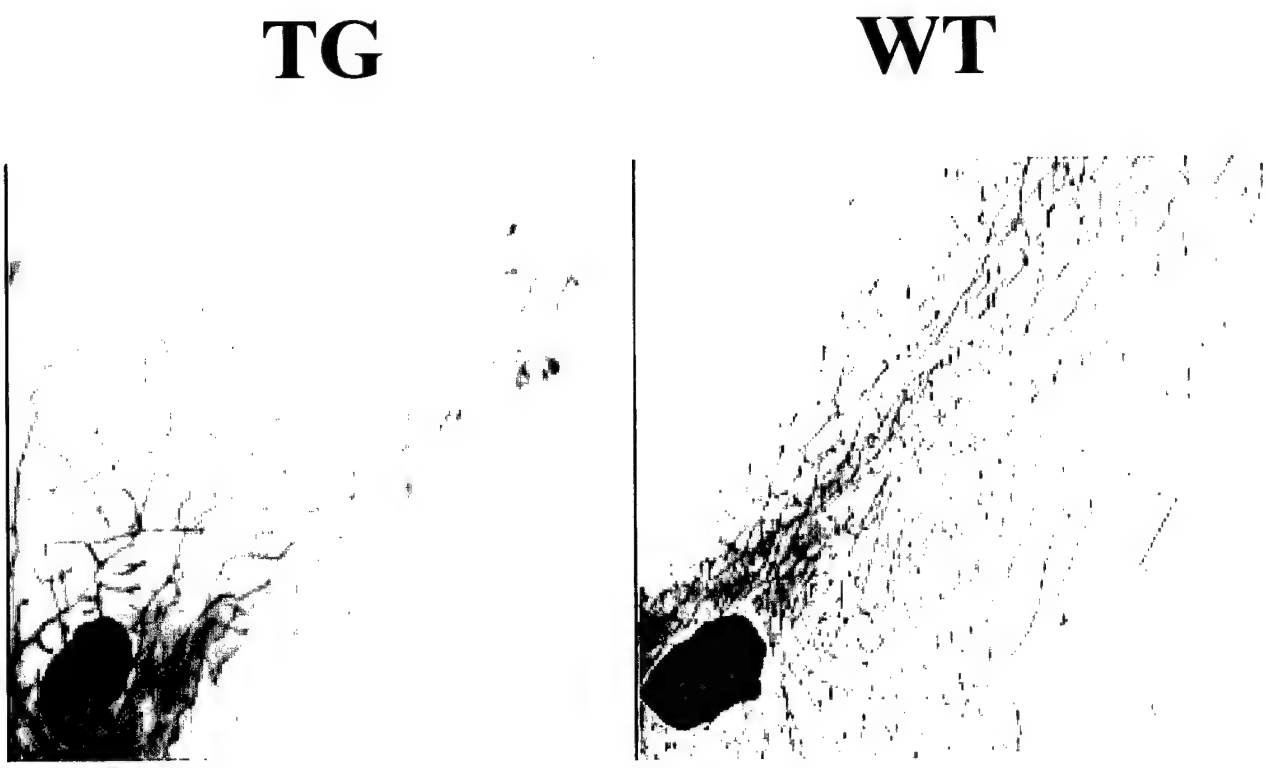


Figure 9: Overexpression of human E6-AP in mouse mammary gland results in impaired mammary gland development. Whole mount analyses of mammary glands from 12 weeks old virgin mice were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG).

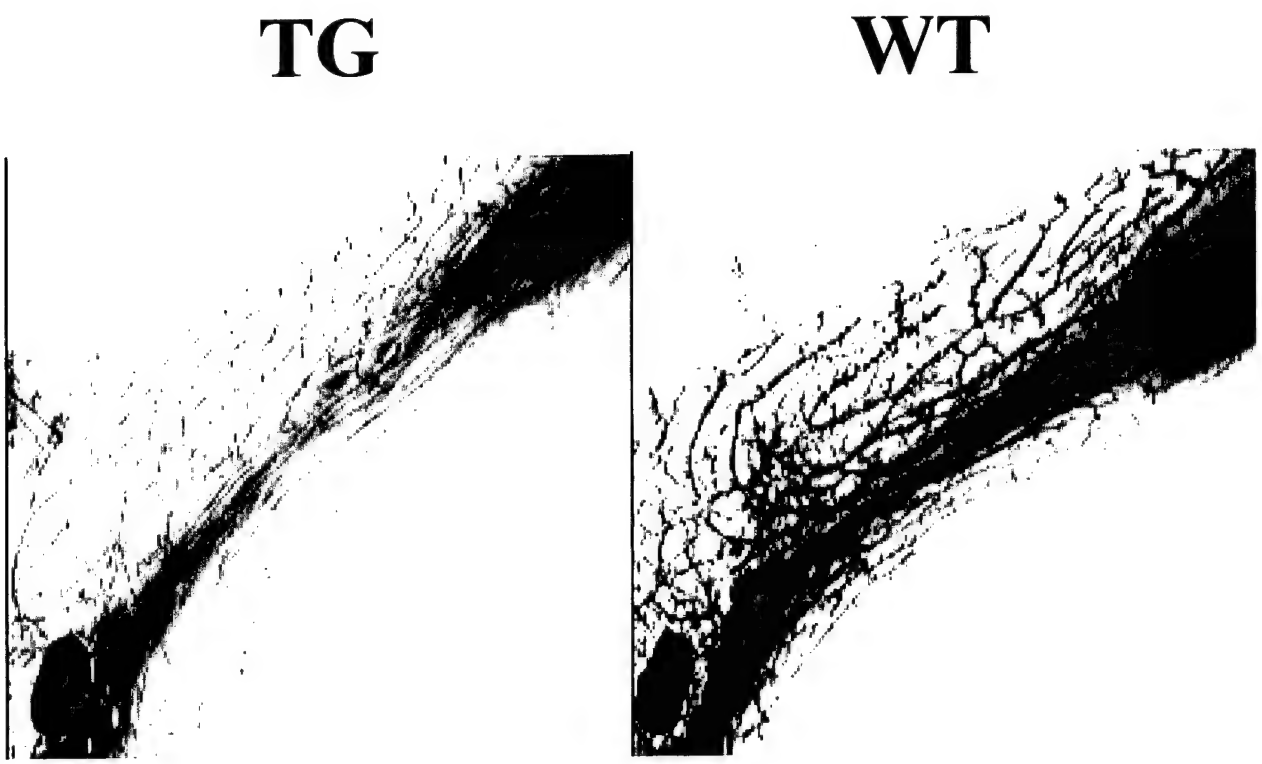


Figure 10: Overexpression of human E6-AP in mouse mammary gland results in impaired mammary gland development. Whole mount analyses of mammary glands from 68 weeks old virgin mice were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG).

A.

B.

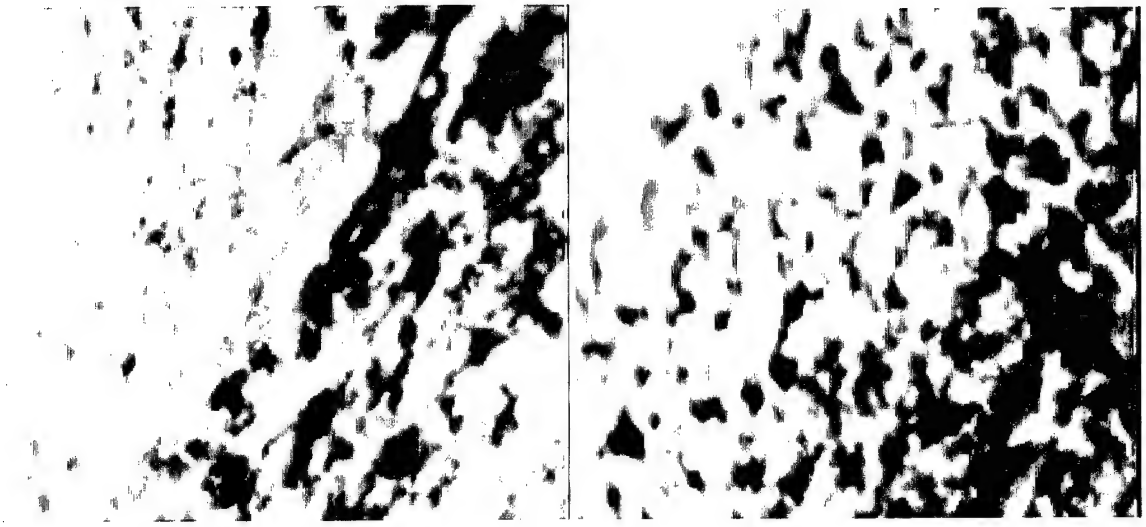
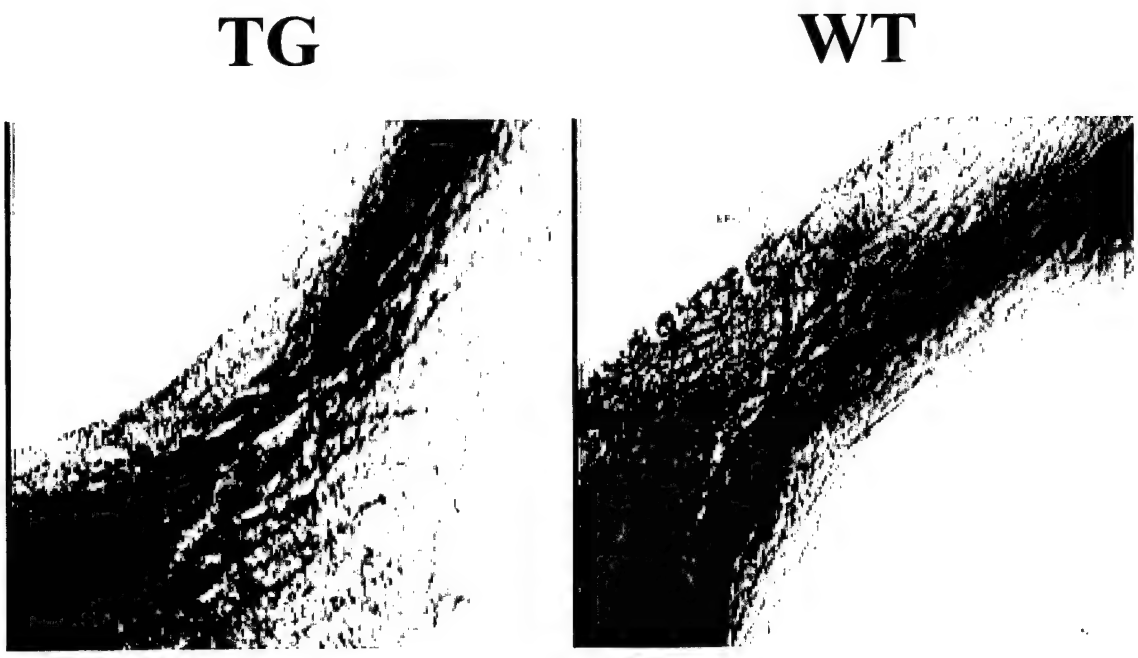


Figure 11: Overexpression of human E6-AP in mouse mammary gland has no significant effect on pregnant mammary glands. Whole mount analyses of mammary glands from 15 days pregnant mammary glands were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG). (A) 5X (B) 20X

A.



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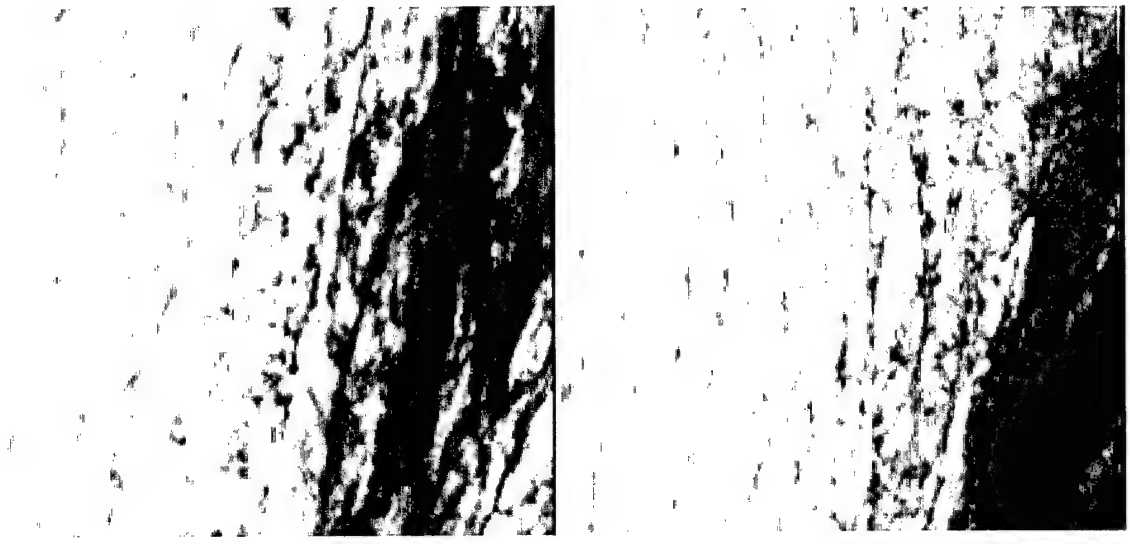

Figure 12: Overexpression of human E6-AP in mouse mammary gland has no significant effect on 15 days involuting mammary glands. Whole mount analyses of mammary glands from 15 days involuting mammary glands were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG). (A) 5X (B) 20X

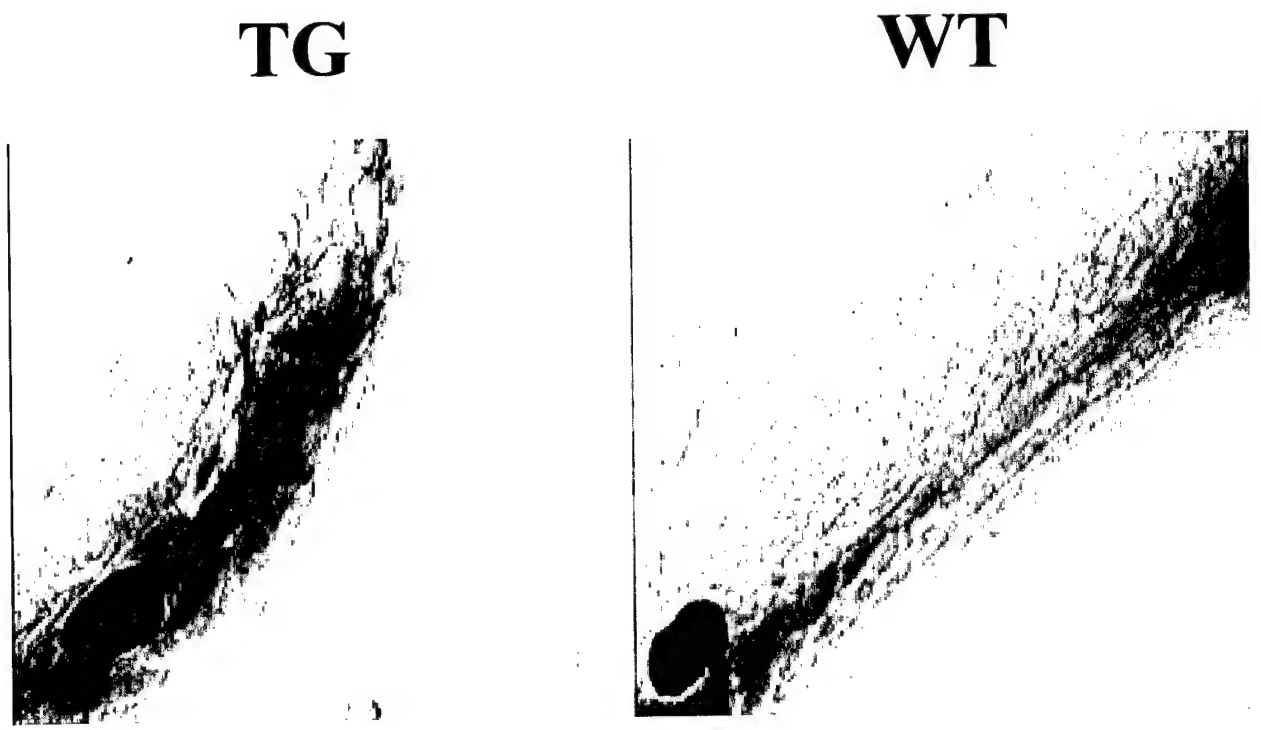

Figure 13: Overexpression of human E6-AP in mouse mammary gland results in smaller mammary glands 8 weeks after involution. Whole mount analyses of mammary glands from 8 weeks involuting mammary glands were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG).

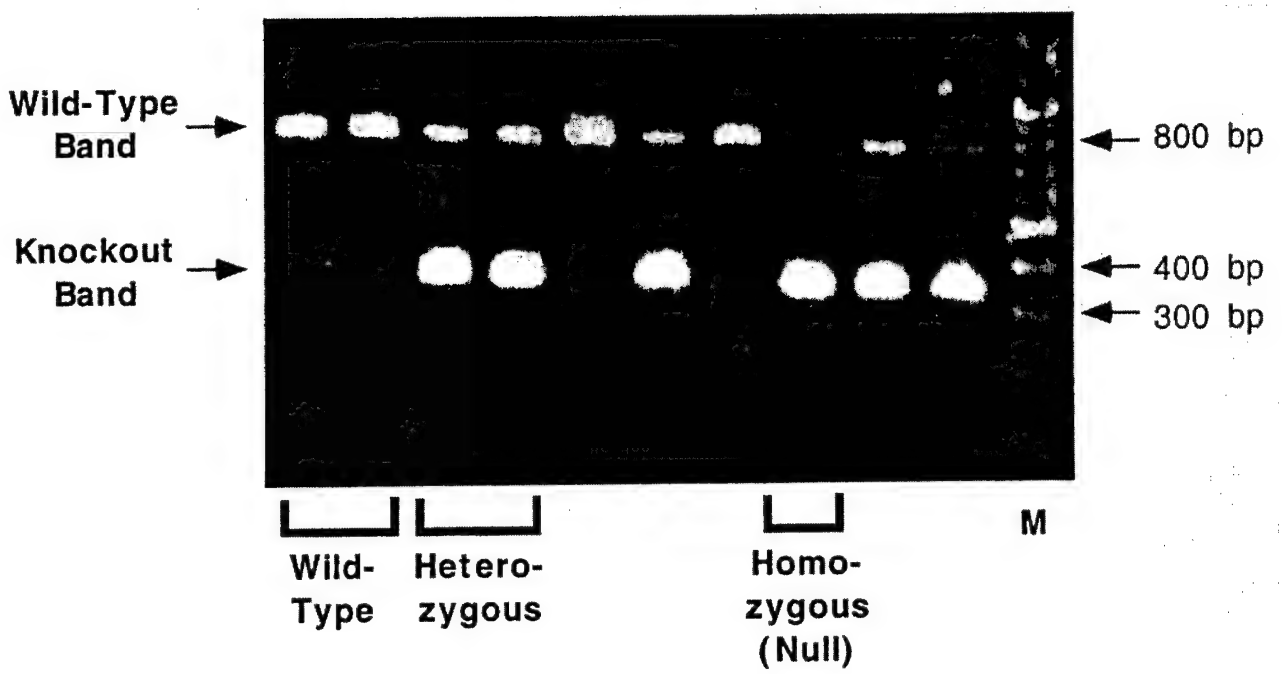

Figure 14: Screening of E6-AP KO (null) animals by PCR method.
In order to identify E6-AP null mice, a PCR screening method was developed. To develop PCR screen 3 pairs of primer sets were designed. These primers, successfully amplify the 750 bp fragment of wild-type E6-AP allele and 350 bp fragment of E6-AP null allele, respectively. The wild-type only generate a 750 bp long band, whereas homozygous E6-AP null animals generate only 350 bp long fragment and heterozygous animals contain both bands.

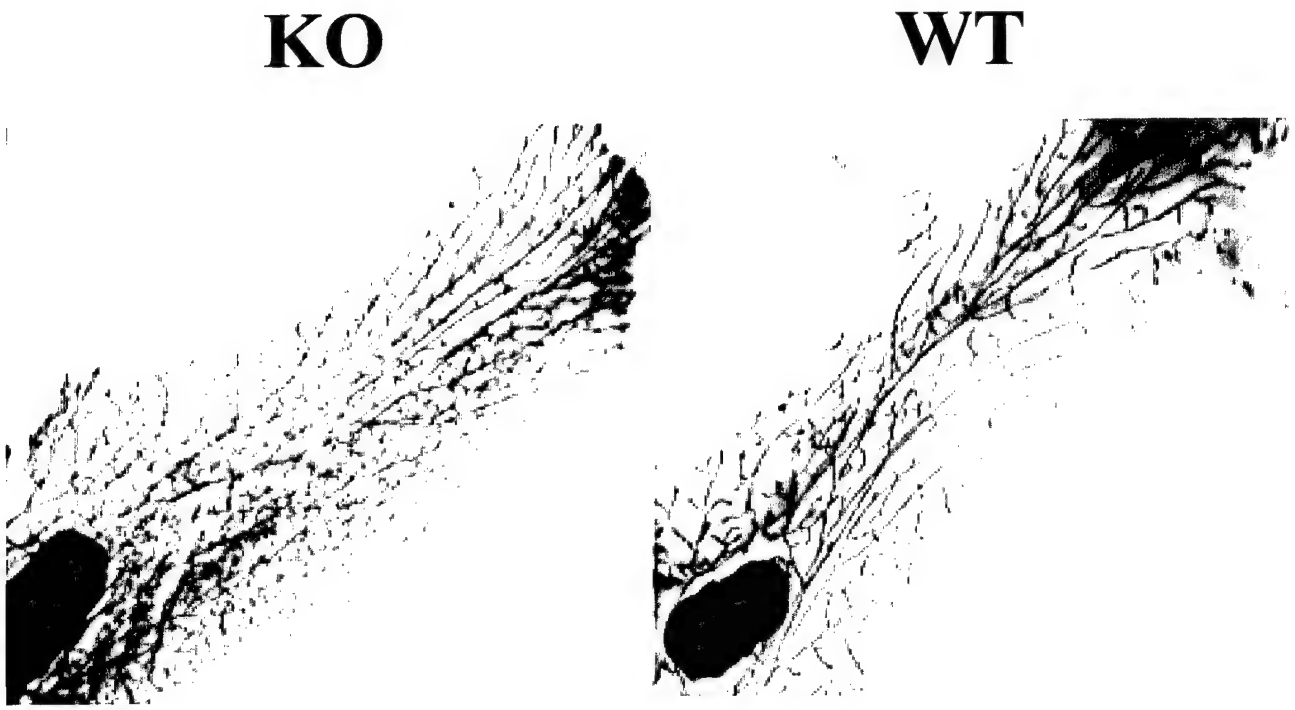

Figure 15: Loss of E6-AP expression results in increased alveolar buds. Whole mount analyses of mammary glands from 8 weeks old virgin mice were performed from wild-type (WT) and E6-AP knockout mice (KO).

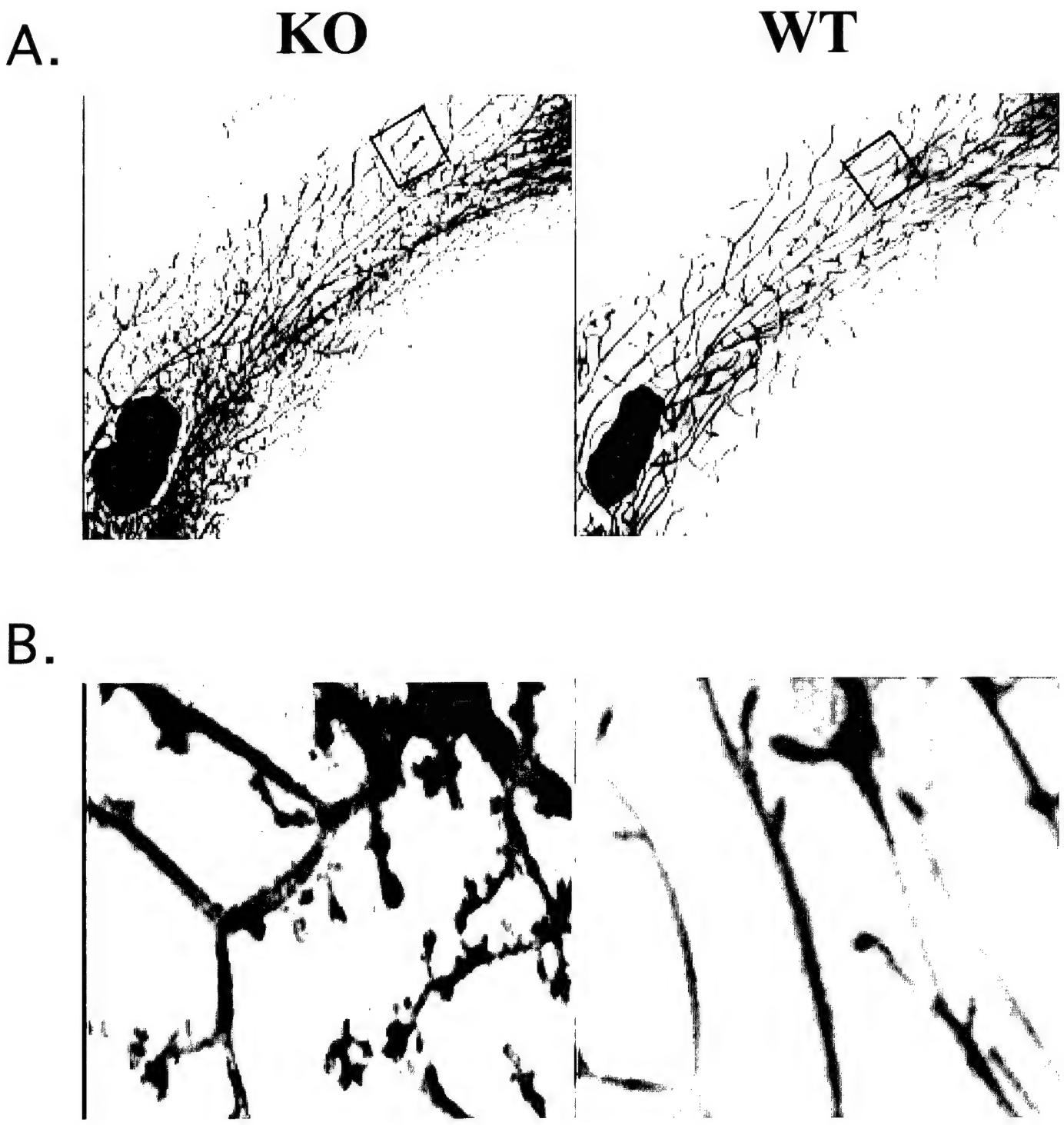

Figure 16: Loss of E6-AP expression results in increased alveolar buds. Whole mount analyses of mammary glands from 12 weeks old virgin mice were performed from wild-type (WT) and E6-AP knockout mice (KO). (A) 5X (B) 20X

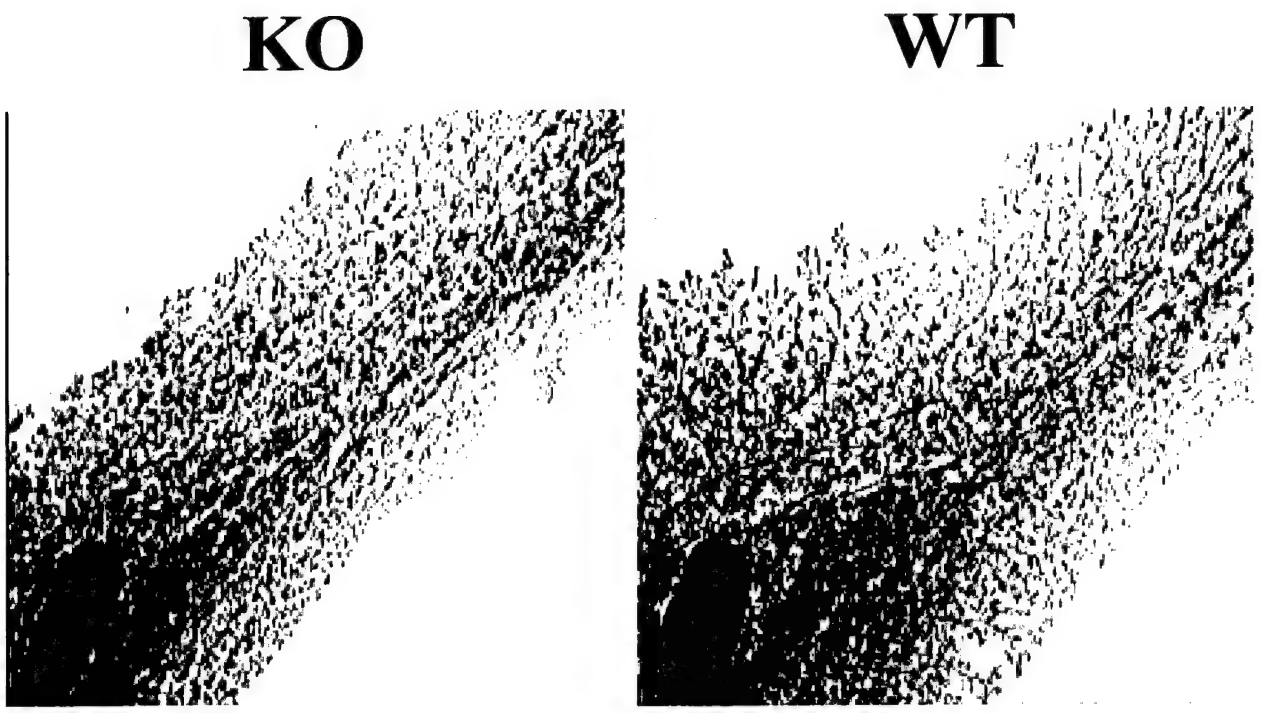

Figure 17: Loss of E6-AP expression has no significant effect on pregnant mammary glands. Whole mount analyses of mammary glands from 15 days pregnant mammary glands were performed from wild-type (WT) and E6-AP knockout mice (KO).

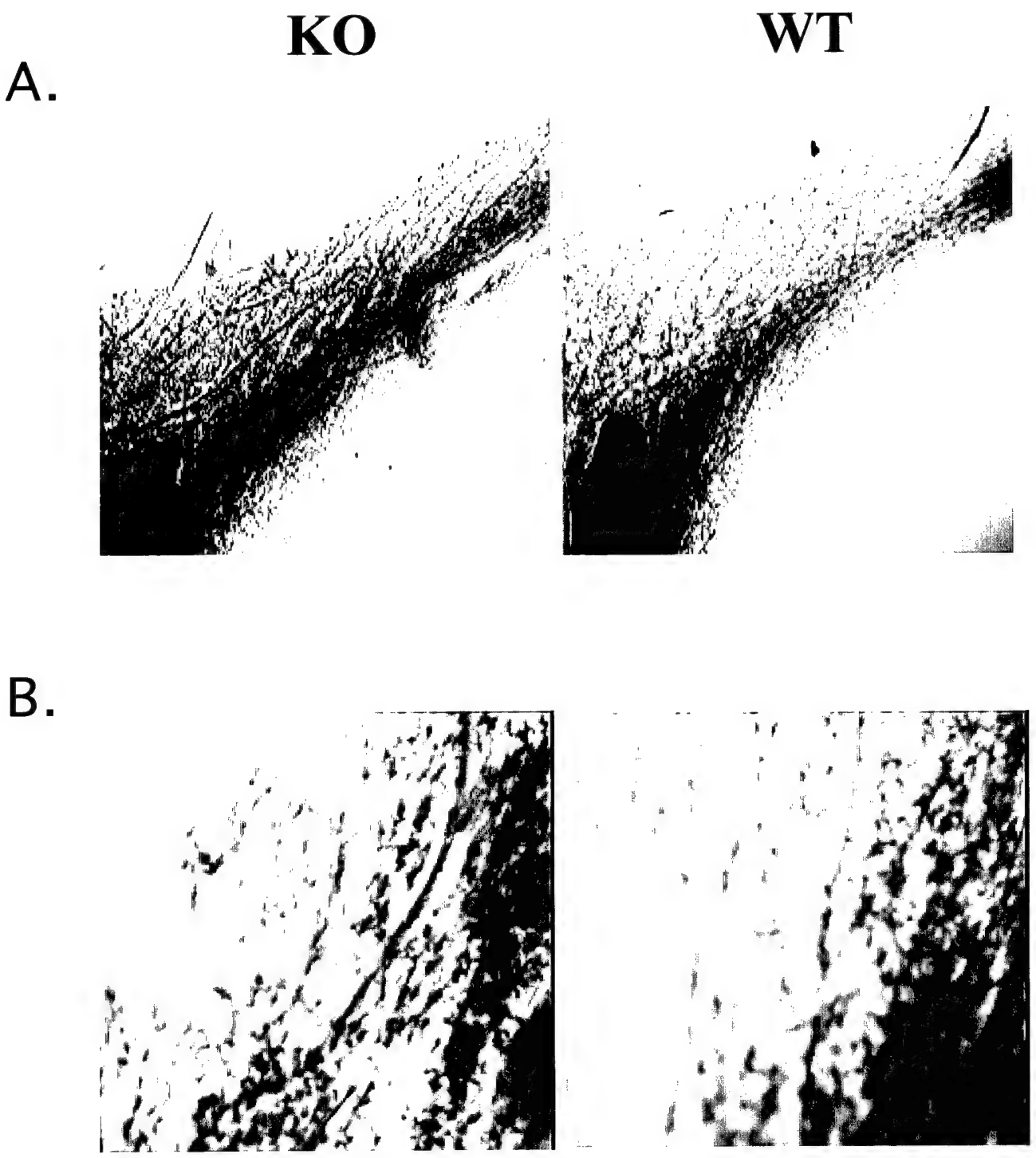

Figure 18: Loss of E6-AP expression has no significant effect on involuting mammary glands. Whole mount analyses of mammary glands from 15 days involuting mammary glands were performed from wild-type (WT) and E6-AP knockout mice (KO). (A) 5X (B) 20X

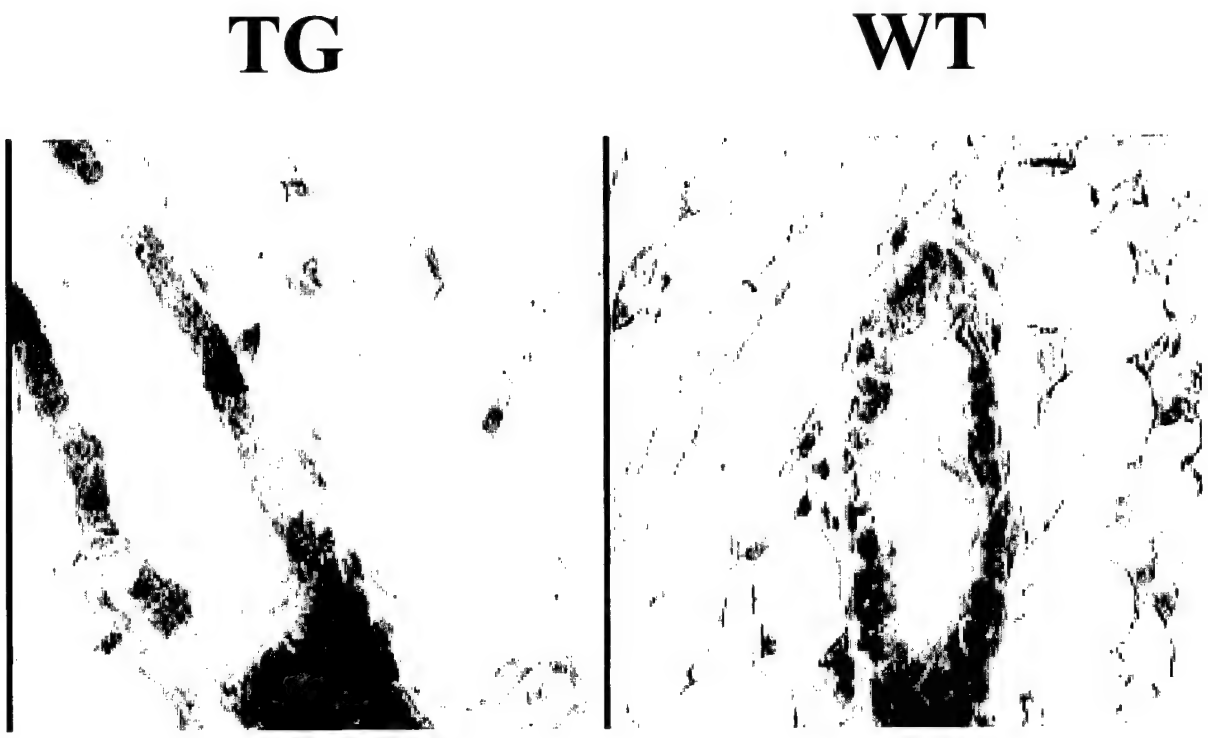

Figure 19: Expression analysis of transgene (MMTV-mutant E6-AP) in the mouse mammary glands. E6-AP expression in the mouse mammary glands was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal is seen as dark (brown) spots. WT, Wild-type mammary gland; TG, ligase-defective mutant E6-AP transgenic mammarygland.

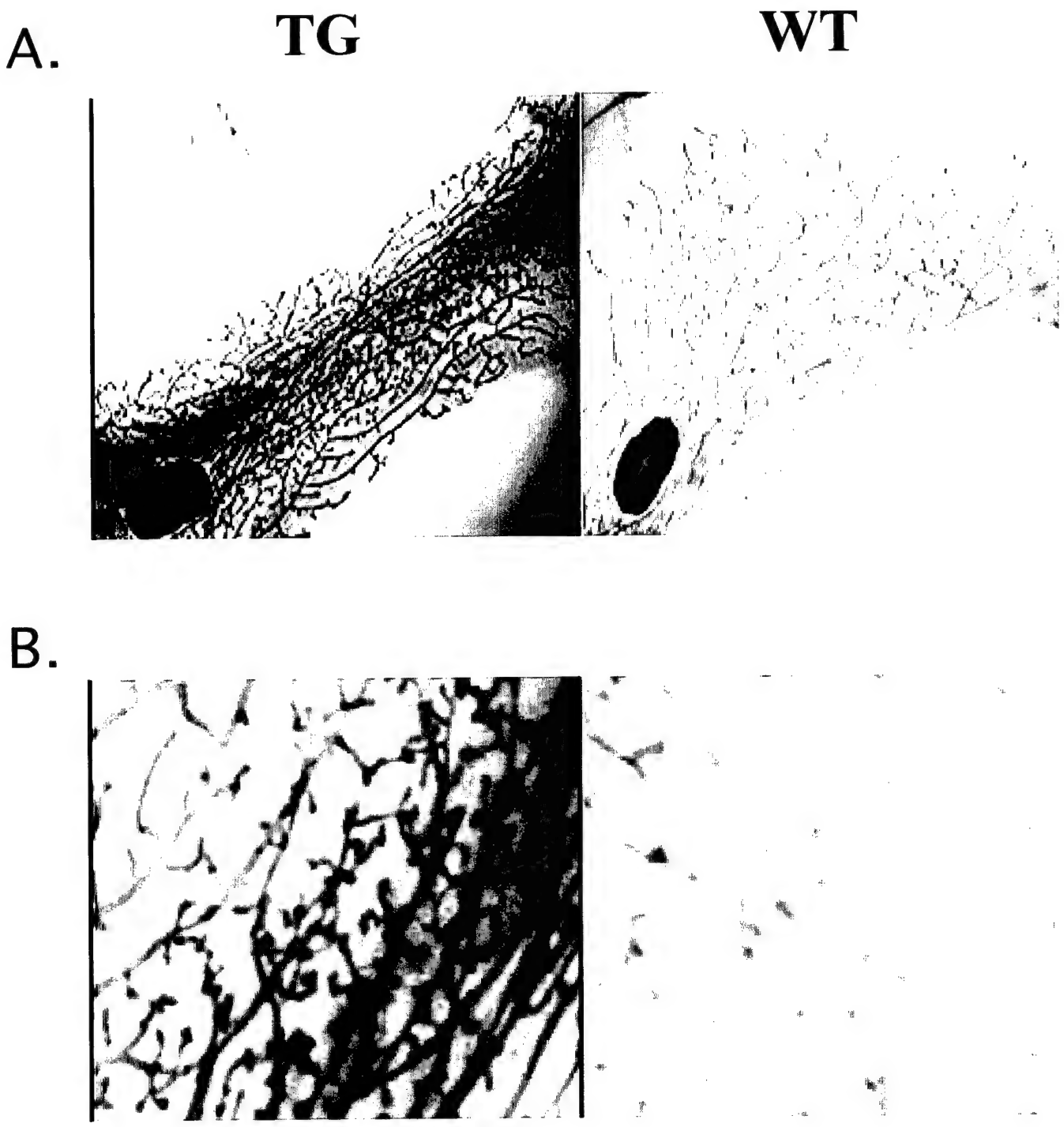

Figure 20: Overexpression of human ligase-defective mutant E6-AP in mouse mammary gland results in increased alveolar buds.
Whole mount analyses of mammary glands from 12 weeks old virgin mice were performed from wild-type (WT) and E6-AP transgenic (TG). (A) 5X (B) 20X

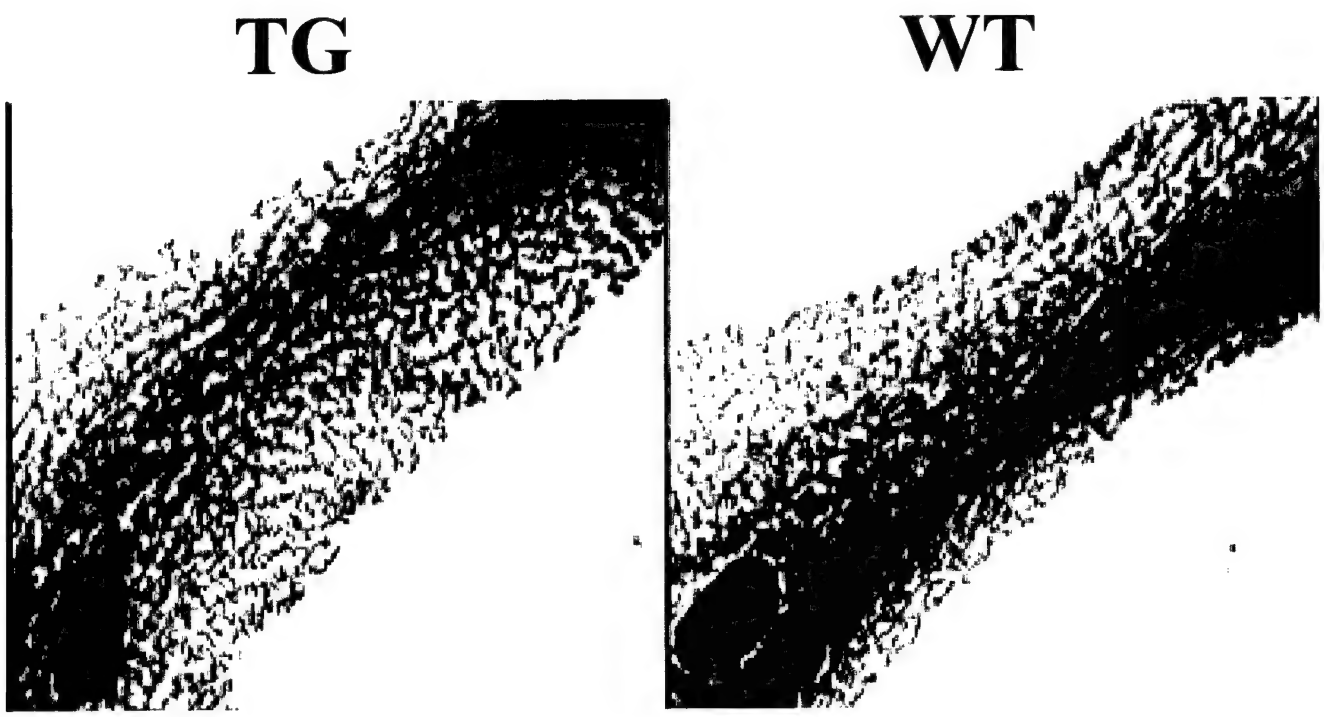

Figure 21: Overexpression of ligase-defective mutant E6-AP in mouse mammary gland has no significant effect on pregnant mammary glands. Whole mount analyses of mammary glands from 15 days pregnant mammary glands were performed from wild-type (WT) and MMTV-mutant E6-AP transgenic mice (TG).

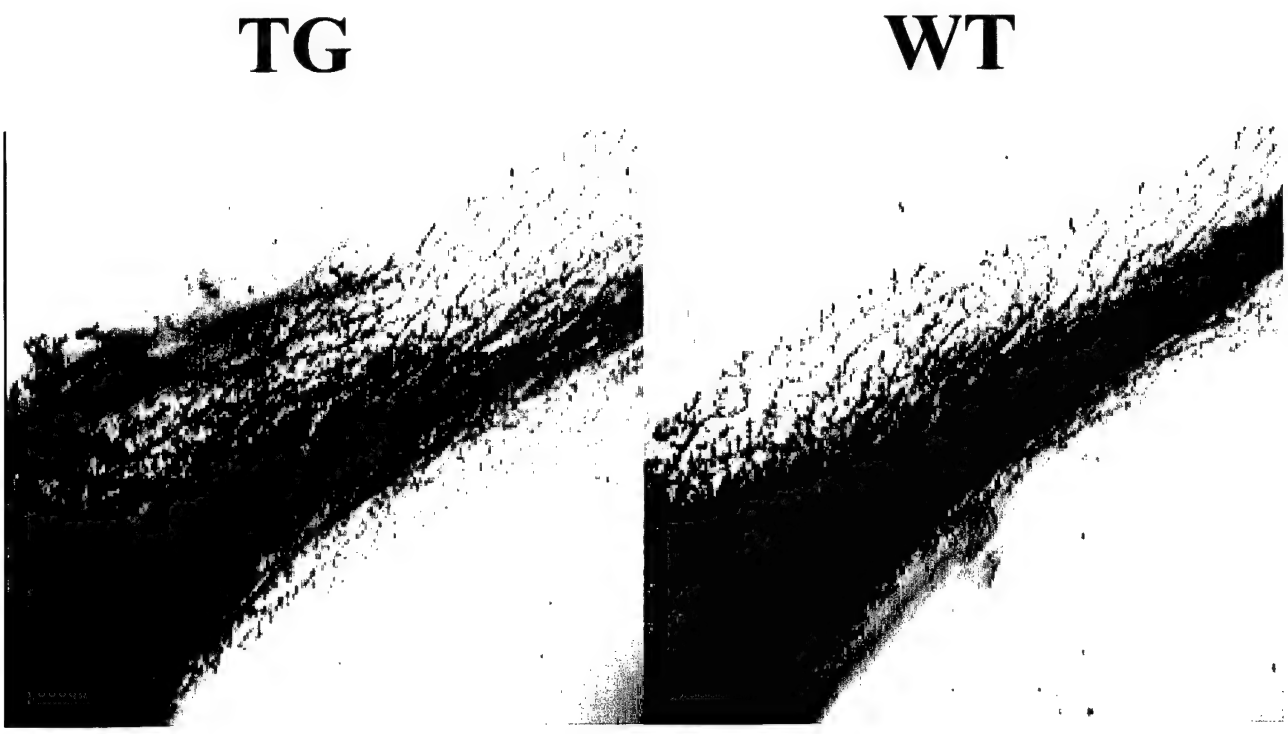

Figure 22: Overexpression of ligase-defective mutant E6-AP in mouse mammary gland has no significant effect on 15 days involuting mammary glands. Whole mount analyses of mammary glands from 15 days involuting mammary glands were performed from wild-type (WT) and MMTV-mutant E6-AP transgenic mice (TG).

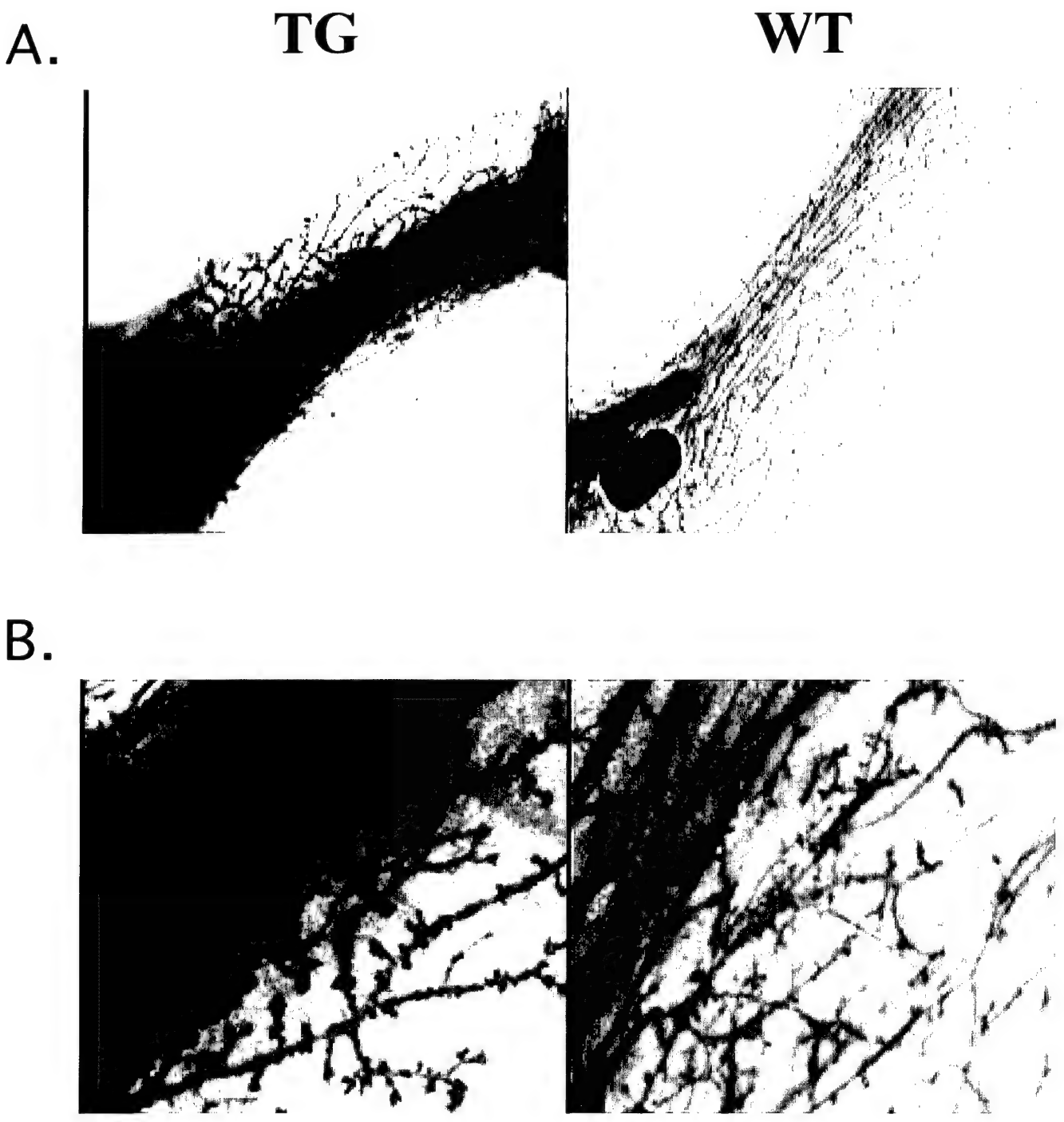

Figure 23: Overexpression of ligase-defective mutant E6-AP in mouse mammary gland results in increased alveolar buds 8 weeks after involution. Whole mount analyses of mammary glands from 8 weeks involuting mammary glands were performed from wild-type (WT) and MMTV-mutant E6-AP transgenic mice (TG). (A) 5X (B) 20X

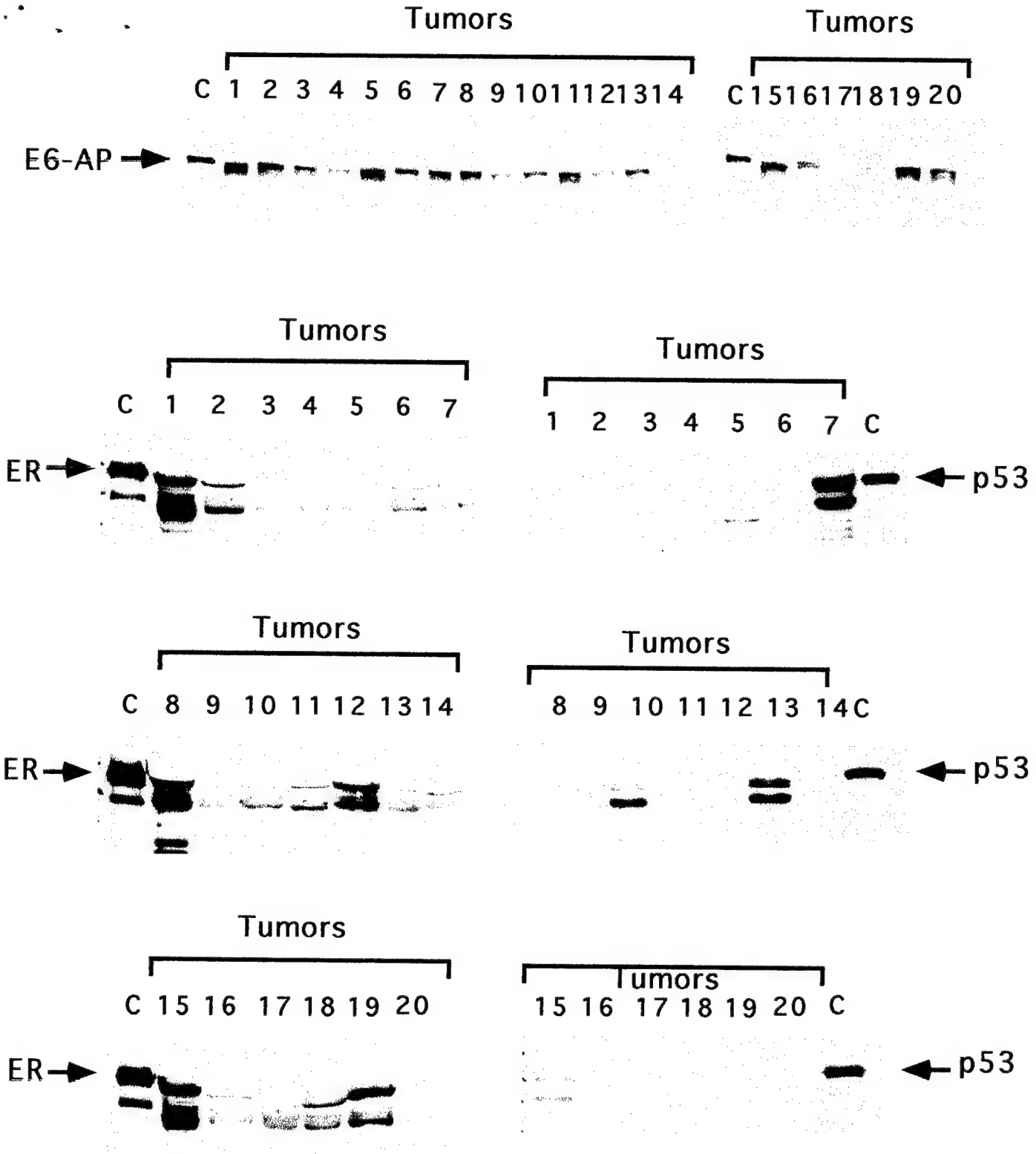

Figure 24: Expression analyses of E6-AP, ER and p53 in human biopsy tumor samples. Tumor samples were collected from the breast cancer center at the Baylor college of Medicine, Houston and expression of E6-AP, ER and p53 were determined by Western blot using E6-AP, ER and p53 specific antibodies.
C, control purified proteins.

Tumor #	E6-AP	ER	Tumor #	E6-AP	ER	Tumor #	E6-AP	ER
1	++++	++++	21	+	+	41	++	+
2	+++	+	22	+++	++	42	++	++++
3	++	-	23	++++	++++	43	+	++
4	+	-	24	+++	+++	44	+	++
5	++++	-/+	25	++	-	45	++	++
6	++	-/+	26	++	++++	46	+++	-/+
7	++	-	27	-/+	-/+	47	+++	-/+
8	+++	+++	28	++	-/+	48	+	-/+
9	+	-/+	29	++	-/+	49	++	-/+
10	++	-/+	30	++	+++	50	++++	+
11	+++	+	31	++++	++++	51	++++	+++
12	++	++	32	++	+	52	++++	+
13	++	-/+	33	+++	++	53	+++	++
14	-/+	-/+	34	+++	+	54	+++	++
15	++++	++++	35	++	+	55	++++	-/+
16	++	-/+	36	+++	-	56	+++	-/+
17	-/+	-	37	+++	++++			
18	+	+	38	+++	+			
19	++++	++	39	++++	+++			
20	++++	-	40	+	++			

Figure 25: Correlation of the expression of E6-AP with that of ER-alpha in breast tumors.
Expression levels of E6-AP and ER-alpha from western blot analysis was artificially graded according to the density of bands. "-" represents negative expression, whereas "-/+" represents very low expression. From "+" to "++++" represent the gradually increasing levels of expression from low to high. Spreaman Rank Correlation Coefficient for the expression of E6-AP with that of ER-alpha is 0.38, p=0.004.

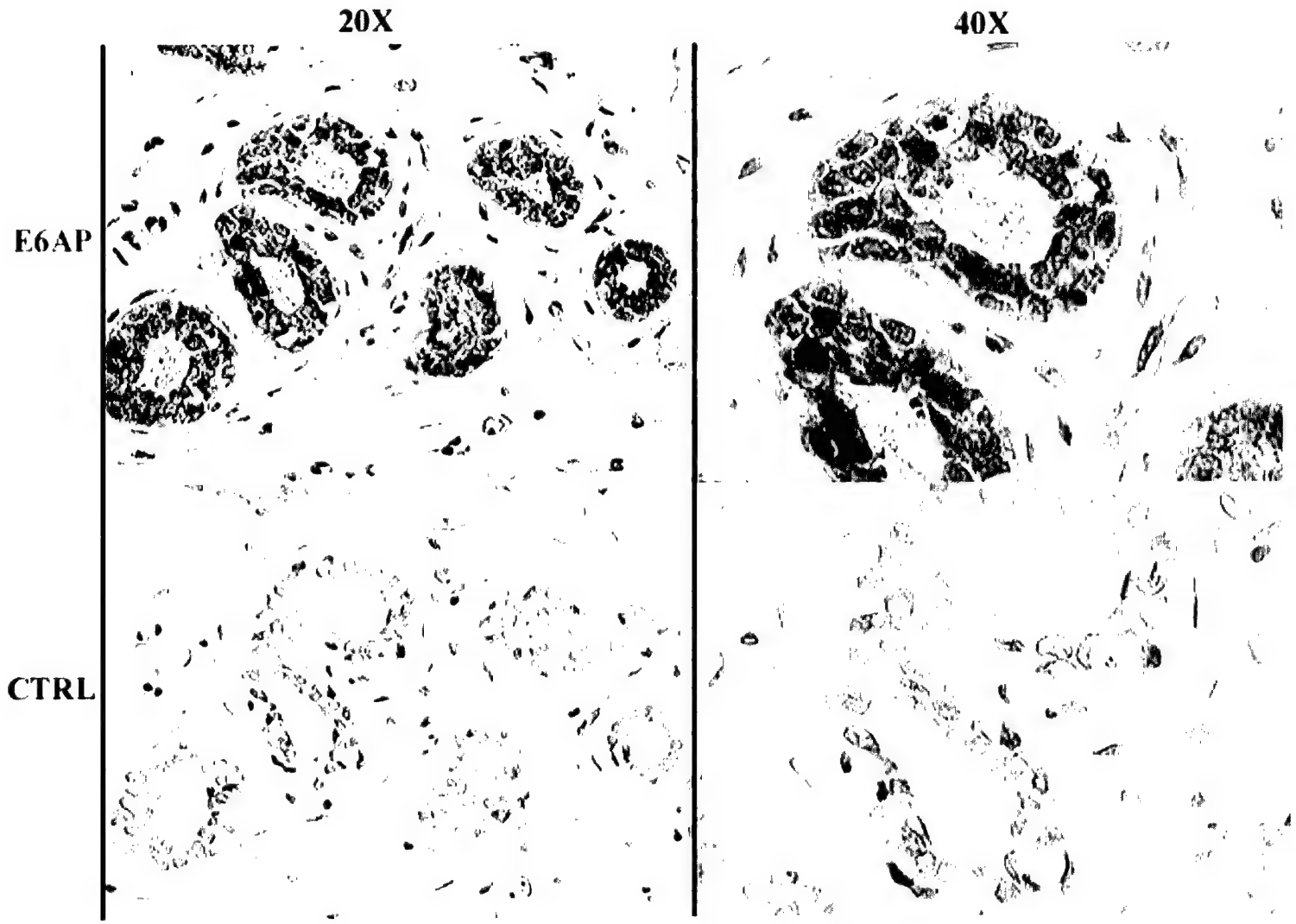

Figure 26: Expression analysis of E6-AP in normal human breast tissues by immunohistochemistry. The expression of endogenous E6-AP was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (brown) spots. E6-AP, E6-AP polyclonal antibody; CTRL, no primary antibody.

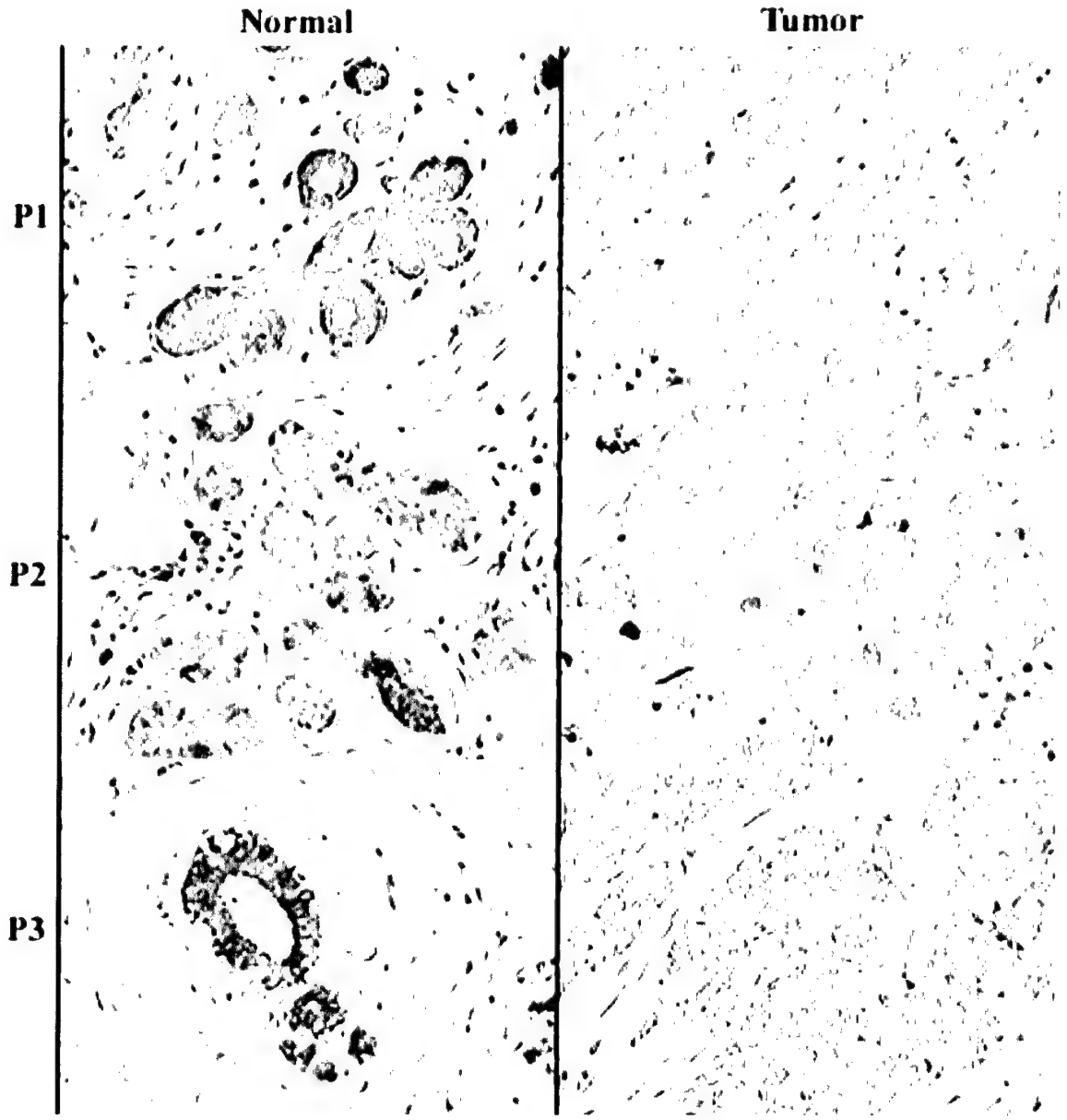

Figure 27: Expression analysis of E6-AP in human breast tumors and adjacent normal tissues by immunohistochemistry. The expression of E6-AP was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (brown) spots. P1, Patient1; P2, Patient 2; P3, Patient 3.

Stages Compared	Rank-sum T	n1,n2-n1	*P* value
SI & SIIB	32	7, 2	<0.01*
SIIA & SIIB	52.5	7, 7	>0.05
SIIIA & SIIB	59.5	6, 1	<0.01*
SI & SIIA	115	9, 5	>0.1
SI & SIIIA	50.5	6, 3	>0.1

Figure 28: Comparison of the expression level of E6-AP between different stages of breast cancer. Expression levels of E6-AP were analyzed in human tumors of different stages by immunohistochemistry. Then the expression of E6-AP was compared within different stages by Wilcoxonrank-sum test. This analysis suggest that E6-AP expression is down in stage IIB with a p value of 0.01.

Appendix 2

1 Abstract submitted to the Endocrine Society
1 Manuscript accepted for publication in Breast Cancer Research, 2002

Please select Print from the file menu to print your Abstract.

The Endocrine Society's 84th Annual Meeting

Filename: 851156
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Awards: Travel Grant Awards.
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Title: E6-ASSOCIATED PROTEIN, E6-AP, IS INVOLED IN THE TUMORIGENESIS OF BOTH MAMMARY GLAND AND PROSTATE GLAND

Xiuhua Gao [1]*, Syed K. Mohsin [2], Thomas M. Weeler [3], Feng Yan [1] and Zafar Nawaz [1].
[1]Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, 77030; [2]Breast Cancer Center, Baylor College of Medicine, Houston, Texas, 77030 and [3]Scott Department of Urology, Baylor College of Medicine, Houston, Texas, 77030.

Nuclear receptor coactivators play a major role in modulation of estrogen (ER) and androgen (AR) receptor functions in breast and prostate, respectively. E6-associated protein (E6-AP) is an E3 ubiquitin-protein ligase involved in the selective protein degradation pathway, ubiquitin-proteasome pathway. It has also been characterized as a coactivator since it can potentiate the transcriptional activity of nuclear hormone receptors, such as ER, AR, progesterone receptor (PR) and glucocorticoid receptor (GR). Based on these facts, we hypothesized that expression of E6-AP may alter during breast/prostate tumorigenesis. To test this hypothesis, we conducted a pilot study to evaluate the expression profile of E6-AP by immunohistochemistry and immunofluorescence. 12 breast cancers (Grade 1 to 3) and 10 prostate cancers (Gleason scores 4 to 6) and their matched adjacent normal breast/prostate tissues were used for the analysis. While high level expression of E6-AP was consistently observed in normal epithelial cells of both mammary gland and prostate glands, decreased expression was found in 8 out of the 12 breast cancers (Chi-Square, $p<0.025$) and 8 out of the 10 prostate tumors (Chi-Square, $p<0.05$). Furthermore, of those tumors which had decreased level of E6-AP, 7/8 breast tumors expressed higher level of ER and 5/8 prostate tumors expressed higher level of AR, in comparison to their respective normal tissues. These data suggested that the alteration of E6-AP expression may have a role in the altered estrogen/androgen action occurring during tumorigenesis. Currently, we are expanding this study to a larger sample size to confirm these

results. We are also planning to establish stably transfected cancer cell lines which overexpress either wild-type or ubiquitin-protein ligase mutant E6-AP. By analyzing these cell lines' growth properties in vitro and their tumorigenicity in vivo, we may know whether the coactivator function or the E3-ubiquitin ligase function of E6-AP is involved in tumorigenesis.

Keyword 1: Steroid receptor coactivator (SRC)
Keyword 2: Ubiquitin
Keyword 3: Tumorigenesis

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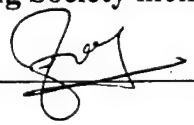

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Breast Cancer Research, 2002

Role of Steroid Receptor Coactivators and Corepressors of Progesterone Receptors in breast cancer

Xiuhua Gao and Zafar Nawaz*
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Abstract

Progesterone, an ovarian steroid hormone, plays a key role in the development and function of mammary gland as it does in uterus and ovary. The action of progesterone is mediated through its intracellular cognate receptor; progesterone receptor (PR), which functions as a transcription factor that regulates gene expression. As the other nuclear receptors, its coregulators, named coactivators and corepressors that are recruited by the liganded or unliganded PR to either enhance or suppress transcription activity, modulate the function of PR. Thus, mutation or aberrant expression of the coregulators would affect PR's normal function and hence disrupt the normal development of mammary gland, which may lead to breast cancer.

Keywords: progesterone receptor (PR), coactivator, corepressor, breast cancer

1. Introduction

The progesterone receptor (PR) is a member of the nuclear receptor (NR) superfamily, which specifically regulates the expression of target genes in response to the hormonal stimulus. In the absence of progesterone, PR is sequestered in a non-productive form associated with heat shock proteins and other cellular chaperones, and, in this state, is considered as being unable to influence the rate of transcription of its cognate promoters [1]. Upon binding with progesterone, the receptor undergoes a series of events, including conformational changes, dissociation from heat shock protein complexes, dimerization, phosphorylation, and nucleu translocation, which enables its binding to progesterone-response elements (PREs) within the regulatory regions of target genes. The binding of PR to the PREs is followed by the recruitment of coactivators and the basal transcription machinery, leading to the upregulation of target gene transcription. There are two forms of PR, named PR-A and PR-B. Detailed molecular dissection has identified two distinct activation function domains (AFs) within the PR. AF-1, which is located in the N-terminal region, is ligand-independent; AF-2, which is ligand-dependent, is contained in the ligand-binding domain (LBD) that is located in the C-terminal region. A DNA-binding domain (DBD) and the hinge region are mapped to the central region.

Coactivators are factors that can interact with NRs in a ligand-dependent manner and enhance their transcriptional activity. Corepressors are factors that interact with NRs and repress their transcription activity. Both types of coregulators are required for efficient modulation of PR's target gene transcription [2]. Therefore, changes in the expression level of PR coactivators or corepressors or mutation of their function domains might affect the transcriptional activity of PR and hence cause disorders of its target tissues, such as mammary gland. This review will describe the coactivators and corepressors that are involved in the transcriptional modulation of PRs, with emphasis on their roles in breast cancer development and progression.

2. Progesterone receptor coactivators

2.1 The SRC family

Abbreviations: ACTR, activator of thyroid hormone; AD, activation domain; AFs, Activation function domain; AIB-1, amplified in breast cancer 1; bHLH, basic helix-loop-helix; BRCA1, breast cancer susceptibility gene 1; CBP, CREB-binding protein; DBD, DNA-binding domain; E6-AP, E6-associated protein; GRIP1, GR-interacting protein 1; HAT, histone acetyltransferase; HMG-1/2, high-mobility group protein-1, 2; LBD, ligand-binding domain; NCoA-1/2, nuclear receptor coactivator 1, 2; N-CoR. nuclear receptor corepressor; NR(s), nuclear receptor(s). PAS, Per-Arnt-Sim domain; p/CIP, p300/CBP cointegrator-associated protein; PNRC1/2, proline-rich nuclear receptor coregulatory protein-1, 2; PR(s), progesterone receptor(s); PREs, progesterone-response elements; RAC3, receptor-associated coactivator 3; RAR, retinoid acid receptor; RID, receptor-interacting domain; RTA, repressor of tamoxifen transcriptional activity; SMRT, silencing mediator of retinoid and thyroid receptor; SRA, steroid receptor RNA activator; SRC, steroid receptor coactivator; TIF2, transcription intermediary factor 2; TR, thyroid receptor; TRAM-1, thyroid receptor activator molecule 1; Uba3, ubiquitin-activating enzyme.

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The SRC (steroid receptor coactivator) family is composed of three distinct but structurally and functionally related members, which are named SRC-1 (NCoA-1), SRC-2 (TIF2/GRIP1/NcoA-2), and SRC-3 (p/CIP/RAC3/ACTR/AIB-1/TRAM-1), respectively. SRC-1 was the first coactivator for the steroid receptor superfamily that was cloned and characterized in 1995 [3]. SRC-2 and SRC-3 were identified thereafter by several laboratories [2]. Sequence analysis of SRC proteins identified a basic helix-loop-helix (bHLH) domain and two Per-Arnt-Sim (PAS) domains in the amino-terminal region. The bHLH/PAS domain is highly conserved among the SRC members and it serves as a DNA binding and protein dimerization motif in many transcription factors [4]. Following the bHLH/PAS domain, there are a centrally located receptor-interacting domain (RID) and a C-terminal transcriptional activation domain (AD). Detailed analysis revealed three conserved LXXLL motifs (NR box) in the RID, that appear to contribute to the specificity of coactivator-receptor interaction. Histone acetyltransferase (HAT) activity was identified in the C-terminal region of SRC members and there also exist activation domains that can interact with the CREB-binding protein (CBP).

All the three members of the SRC family interact with PR and enhance its transcriptional activation in a ligand-dependent manner [5, 6]. Targeted deletion of SRC-1 gene in mice has indicated that SRC-1 is important for the biological actions of progesterone in mammary gland development since the hormone-induced ductal elongation and alveolar development is greatly impaired in the null mice [7]. In the meantime, the expression of SRC-2 mRNA was elevated in SRC-1 null mice, suggesting that SRC-2 can partially compensate for SRC-1 function [7]. SRC-3 is the most distinct one among the three members, since it coactivates not only the nuclear receptors but also other unrelated transcription factors such as those in the cAMP or cytokine pathways. Compared with the widespread expression of SRC-1 and SRC-2, expression of SRC-3 is restricted to the mammary gland, and several other tissues [8]. Disruption of SRC-3 gene in mice causes severe growth and reproductive defects, including the retardation of mammary gland development [9]. Furthermore, amplification and overexpression of SRC-3 were observed in 10% and 64% of human primary breast cancers, respectively [10], which indicated that SRC-3 is not only essential for the normal mammary development, but also plays a role in breast tumorigenesis.

2.2 E6-AP/RPF-1

E6-AP, E6-associated protein, and RPF1, the human homolog of yeast RSP5, are E3 ubiquitin-protein ligases that target proteins for degradation by the ubiquitin pathway. Besides, they are also characterized as coacitvators of steroid receptors. It has been demonstrated by transient transfection assay that RPF1 and E6AP could potentiate the ligand-dependent transcriptional activity of PR, GR, and other nuclear receptors [11, 12], and furthermore, they acted synergistically to enhance PR transactivation [12]. Additionally, the coactivation function of E6-AP and RPF1 are seperable from the E3 ubiquitin-protein ligase activity, as ubiquitin ligase-defective E6-AP and RPF1 exhibited normal coactivation function.

E6-AP is expressed in many tissues including mammary gland. From its ability to coactivate PR and ER in a hormone-dependent manner, it was assumed that E6-AP is an essential regulator for the development of normal mammary gland and mammary tumors. The first evidence of a relationship between E6-AP and breast cancers was obtained from the study of a spontaneous mouse mammary tumorigenesis model, which demonstrated that E6-AP was overexpressed in tumors when compared with normal tissues [14]. Recently, we examined the expression pattern of E6-AP in biopsy samples of human breast cancers, and our results suggested that E6-AP expression was decreased in tumors in comparison to the adjacent normal tissues [Gao et al, unpublished data]. Furthermore, we demonstrated that the decreased expression of E6-AP was stage-dependent, and the expression of E6-AP was inversely correlated with that of ER in breast tumors. Since ER plays a major role in breast cancer development, and PR is a target of estrogen, the changes of the expression level of E6-AP might interfere with the normal functioning of ER and PR, hence participating in the formation and progression of breast tumors.

2.3 SRA

The growing family of nuclear receptor coactivators has recently aquired a unique member, that is steroid receptor RNA activator (SRA) [14]. Differing from the other coactivators, SRA functions as a RNA transcript instead of as a protein and specifically coactivates the transcriptional activity of steroid receptors, including PR, ER, GR, and AR. It was demonstrated that SRA exists in a

ribonucleoprotein complex containing SRC-1 and it mediates transactivation through the AF-1 domain that is located at the N-terminal region of nuclear receptors, which makes it different from the other coactivators.

SRA is expressed in normal and malignant human mammary tissues [15, 16]. Compared with the adjacent normal region, elevated expression of SRA was found in tumors [16]. Although it is currently unknown whether the expression of SRA is correlated with that of PR or ER, the increase in the SRA levels in tumor cells may contribute to the altered ER/PR action, which is known to occur during breast tumorigenesis.

2.4 L7/SPA

L7/SPA is a 27kDa protein containing a basic leucine zipper domain. L7/SPA is an antigonist specific transcriptional coactivator because it can only potentiate the partial agonist activity of some antigonists, including tamoxifen and RU486, but has no effect on the agonist-mediated transcription [17]. This unique property of L7/SPA suggests that it might play a role in the development of resistance to hormone therapy for breast cancers.

2.5 CBP/p300

CREB-binding protein (CBP) was initially characterized as a coactivator required for efficient transactivation of cAMP-response element-binding protein, and p300 was first identified as a coactivator of the adenovirus E1A oncoprotein. CBP and p300 share many functional properties in that both of them function as coactivators for multiple NRs as well as p53 and NF-kB [3], both possess intrinsic HAT activity and both can recruit HAT and p/CAF (CBP/p300-associated factor) [18]. Besides, CBP/p300 interacts with members of SRC family and synergizes with SRC-1 in the transactivation of ER and PR [19].

2.6 Others

In addition to the coactivators discussed above, there are a few other proteins that have been demonstrated to be able to upregulate the transcriptional activity of PR. HMG-1/2 (the chromatin high-mobility group protein-1, 2), TIP60 (Tat-interacting protein), PNRC1/2 (proline-rich nuclear receptor coregulatory protein-1, 2), Cdc25B, and GT198 are all functioning as PR coactivators, as demonstrated by transient transfection assays. In terms of its roles in breast cancer development, Cdc25B was prominent among these coactivators, because Cdc25B transgenic mice exhibited mammary gland hyperplasia and increased steroid hormone responsiveness [20]. The significance of all these coactivators in vivo needs to be further investigated.

3 Progesterone Receptor Corepressors

3.1 N-CoR/SMRT

Nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid receptors (SMRT) are both corepressors of numerous transcription factors, including steroid hormone receptors. Both N-CoR and SMRT interact with the nuclear receptors through the RIDs located in the C-terminal portion of the proteins, while their transcriptional repression domains were mapped to the N-termini [21]. N-CoR/SMRT also associate with HDAC3 in large protein complexes, which are an important pathway for transcriptional repression. Corepressors N-CoR and SMRT interact with the NRs either in the absence of agonists (in the case of TR and RAR), or in the presence of antigonists (in the case of steroid receptors) [21]. Since N-CoR and SMRT are common corepressors for transcription factors, slight alteration of their expression level in certain tissues might result in significant transcrptional changes, leading to altered development of mammry gland, even tumors.

3.2 BRCA1

BRCA1 is a breast cancer susceptibility gene, and its inherited mutations are correlated with an increased risk of breast and ovarian cancer [22]. The role of BRCA1 in cancer development is unclear. In addition to its ability to coactivate p53 and to modulate p300/CBP expression, BRCA1 was also demonstrated as a ligand-independent corepressor for ER, AR and PR [23]. When BRCA1 is mutated, all of these pathways will be more or less impaired, therefore, the effect of BRCA1 in cancer development might be multiplex.

3.3 Others

Ubiquitin-activating enzyme 3, Uba3, is the catalytic subunit of the activating enzyme in the ubiquitin-like NEDD8 (neural precursor cell-expressed developmentally down-regulated) conjugation (neddylation) pathway. Recently, Uba3 was demonstrated as a corepressor of ER, AR and PR in mammalian transfection assays [24]. Uba3 inhibited the transactivation of ER in a time-dependent manner and neddylation activity

of Uba3 is required for this suppression, suggesting that Uba3 suppresses steroid receptor activity by promoting the termination of receptor-mediated gene transcription rather than interfering with the initial events.

RTA, repressor of tamoxifen transcriptional activity, has recently been defined as a potent repressor of tamoxifen–mediated ERα transcriptional activity as well as the agonist activities of ERβ, GR, and PR [25]. The interaction of RTA with the NRs requires the participation of RNA, as mutation of the RRM (RNA recognition motif) in RTA compromises its ability to repress transcription [30].

The roles of Uba3 and RTA in mammary gland development and tumorigenesis await for further investigation.

4. Summary

As a transcription factor, PR activates target gene transcription in response to the hormonal stimulus, and its functions are modulated by coactivators and corepressors. The coactivators and corepressors of PR identified so far are not PR specific, since they can also modulate the transactivation of many other NRs. Different coregulators exert their actions through different mechanisms, and involvement in the development of normal mammary gland and the formation or progression of tumors has been reported in some coactivators and corepressors. However, further studies are required to reveal the exact roles of the coactivators and corepressors in mammary gland tumorigenesis before any conclusion can be made.

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